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The mechanism of action of helper T cells in the induction of delayed-type hypersensitivity

by

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ABSTRACT

Culture conditions have been established that allow the induction of DTH precursor cells, present in a cell population derived from unsensitised spleen cells, only when antigen-specific, radioresistant, Thy-1 bearing helper cells are added. This specific cellular cooperation acts via the linked recognition of two determinants on the antigen; thus cells primed to the protein antigen fowl gamma globulin (FGG) will only allow the induction of DTH reactivity against the second antigen, burro red blood cells (BRBC), if the conjugate FGG-BRBC is present in the cultures. The requirement for physical linkage between the two antigens has been demonstrated by the observation that DTH to BRBC is induced when the conjugate FGG-BRBC is present and not when BRBC and FGG are given as uncoupled antigens.

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LIST OF ABBREVIATIONS

ag:	antigen
B cells:	bursa- and/or bone marrow-derived cells
BRBC:	burro red blood cells
CMI:	cell-mediated immunity
CNBr:	cyanogen bromide
CRBC:	chicken red blood cells
DNFB:	dinitro-fluorobenzene
DNP:	dinitro-phenyl
DTH:	delayed-type hypersensitivity
FCS:	fetal calf serum
FGG:	fowl gamma globulin
FGGap:	alum-precipitated fowl gamma globulin
FGGBRBC:	fowl gamma globulin physically coupled to burro red blood cells
FGGMRBC:	fowl gamma globulin physically coupled to mouse red blood cells
(H,G)-A--L:	(His,Glu)-Ala--Lys
HRBC:	horse red blood cells
ip:	intraperitoneal(ly)
iv:	intravenous(ly)
KLH:	keyhole limpet hemocyanin
Ly:	lymphocyte antigen series
MEM:	minimum essential medium
MHC:	major histocompatibility complex
MRBC:	mouse red blood cells
PEC:	peritoneal exudate cells

PFC:	plaque-forming cells
(Phe,G)-A--L:	(Phe,Glu)-Ala--Lys
PPD:	purified protein derivative of tuberculin
RBC:	red blood cell
sc:	subcutaneous(ly)
SRBC:	sheep red blood cells
T cells:	thymus-derived lymphocytes
(T,G)-A--L:	(Tyr,Glu)-Ala--Lys
Thy-1:	thymus-derived cell marker (0)
WBC:	white blood cell

I. INTRODUCTION

1. Historical Introduction

The main purpose of the present study is to establish the requirements for the induction of a delayed type cell-mediated immune response. If one is to gain an understanding of immunoregulation, one must know the requirements for the induction of particular classes of immune response, and how these are related to one another. Much work has been done on the requirements for induction of other classes, but relatively little is known about the specific induction of DTH reactivity. I should like to briefly review the major events in immunological history which led to the recognition of delayed type hypersensitivity (DTH) as a class of immune response emphasising its similarities and differences from other classes of immune reactivity.

The first recorded description of an induced delayed-type hypersensitivity reaction was by Jenner in 1798 when he described the "reaction of immunity" in people revaccinated against the Pox virus. These people were reported to react with "a papular erythematous lesion of the skin reaching its maximum in 24-72 hours after vaccination and disappearing without passing through the pustular stage or leaving a scar" (as published by Turk, 1980). The first use of hypersensitivity reactions as a diagnostic test was by Koch (1890) when he reported the reactions of tuberculous patients following a subcutaneous injection of tuberculin. When it was realised that immune responses to foreign antigens such as injected tuberculin could actually be responsible for tissue damage to the individual concerned, the term "hypersensitivity" was introduced (Portier and Richet,1902). These investigators were not actually looking at delayed hypersensitivity reactions, but at immediate hypersensitivity associated with anaphylactic shock. The term "allergy" was introduced to denote the altered capacity of pre-exposed individuals to react to skin injections of a given reagent compared to those who had not been previously infected (von Pirquet, 1906). It was not until 1921 that "bacterial allergy" as it was then called, was recognised and described as two distinct reactivities (Zinsser, 1921). An immediate skin reactivity consisting of a wheal which appeared 2-15 minutes after injection of antigen and lasted 30-120 minutes without appreciably damaging local tissues was associated with antibody-mediated immunity and anaphylaxis. A distinct reaction to antigen injection was characterised by no immediate effect but, beginning about 4-5 hours after injection, a swelling became apparent which might not peak until 48 hours after injection and was associated with inflammation and some cell death. It was discovered a few years later that these types of reactivity could be induced against agents other than pathogenic organisms when Dienes and Schonheit (1926) treated tuberculous guinea pigs with egg white and subsequently induced hypersensitive, delayed and prolonged skin reactivity with tiny amounts of egg white administered subcutaneously. They associated the establishment of the later reaction with the tuberculous infection, partly because the strongest delayed reactions were produced by injecting antigen directly into the tuberculous focus, while non-tuberculous guinea pigs treated similarly only developed immediate sensitivity reactions or serum precipitins specific for the egg white.

The gross and histological differences between the two hypersensitive reactivities were reported by Dienes and Mallory (1932). The immediate reaction was characterised by rapidly developing edema and rapid intense infiltration of polymorphs; while the delayed reaction was characterised by a slowly developing exudation with early infiltration by mononuclear cells and few polymorphs. Landsteiner and Chase (1941) injected guinea pigs with picryl chloride intraperitoneally together with killed tubercle bacilli and demonstrated a delayed skin reaction to the picryl chloride upon subcutaneous injection and contact sensitivity upon dermal application. This was the first time contact sensitivity had been associated with the immune reactivity of sensitive animals. Landsteiner and Chase (1942) then demonstrated that this reactivity could not be passively transferred to other guinea pigs by serum antibodies, but by peritoneal exudate cells. They also demonstrated that the cells had to be alive to transfer the reactivity, since "moderate heating sufficient to kill the exudate cells abolishes the effect".

All of this evidence seemed to differentiate between humoral or antibody-mediated immunity and cell-mediated immunity on the basis of requirements for passive transfer of the reactions to other animals, the kinetics and other gross characteristics of skin reactivity following subcutaneous injection of antigen, and the histology of the lesions induced in the two types of hypersensitive reactions. Nothing was yet known about the mechanism of induction of the different states except that the only way to induce delayed hypersensitivity appeared to be the injection of the antigen into a tuberculous focus or mixed with an adjuvant containing the killed tubercle bacillus (Freund and McDermott, 1942). Karush and Eisen (1962) maintained that delayed-type hypersensitivity was really due to the presence of small amounts of very high affinity antibody which may be taken up on the surface of other cells, such as macrophages or lymphocytes, thus invoking essentially the same mechanism for the induction of both types of immunity but postulating a qualitative difference in the type of antibody produced. The antibody would indeed have to be of a new category, since it had already been shown that agammaglobulinemic human patients could mount DTH responses (Good, Bridges, Zak, and Pappenheimer, 1959).

About this time, the problems of histoincompatibility were beginning to be appreciated as the peritoneal exudate cells, transferred from one animal to another, were themselves rejected before the experimenters could determine whether these cells might continue to be stimulated to eventually form detectable antibody or whether they acted to produce only cell-mediated responses. Some inbred mouse lines were being developed and the passive transfer of immunity to tumour grafts within these lines was being studied. Mitchison (1954) reported that transplantation immunity was not passively transferrable by serum but only by living lymphoid cells. Muscle cells were not efficient at transferring the immunity, and of all lymphoid cells, only cells from the specific draining lymph nodes were able to transfer immunity effectively. A study of the kinetic appearance of the activity in the draining lymph nodes showed the immune capability appeared about 4 days after tumour grafting, peaked about 10 days later and had waned by 20 days. Billingham, Brent and Medawar (1955) were also studying transplantation immunity by analysing the acceptance or rejection of skin grafts between inbred lines of mice. They noted that immunity could only be transferred by living lymphoid cells, those of the draining lymph nodes being particularly effective; the accelerated rejection of second grafts, even in those mice with adoptively acquired immunity; and the similarities between tumour or skin graft immunity and the tuberculin and sensitisation reactions of the delayed type. These similarities were further emphasised by Brent, Brown and Medawar (1958) in their reports of skin transplants between guinea pigs. Delayed immune reactions against skin homografts were demonstrated by two different techniques:

i) the "direct reaction" involving the injection of donor type antigen subcutaneously into animals bearing a donor type graft; and

ii) the "transfer reaction" involving the injection of living cells, from the draining lymph node of an animal bearing the graft, subcutaneously into the graft donor.

Brent and Medawar (1963) also noticed that the intradermal injection of normal lymphocytes from one guinea pig into a number of possible outbred donors of homografts elicited delayed hypersensitivity reactions of unequal intensity providing an almost exact forecast of the intensity of rejection of a skin transplant from each of those donors. These observations appear to show that a background state of immunity, specifically DTH in this instance, directed against alloantigens, already existed in these animals before intentional antigen-specific priming.

Raffel and Newel (1958) reported reactivity in outbred guinea pigs following the intradermal injection of low doses of antigen (egg albumin) alone, or even lower doses of antigen-antibody complexes in adjuvant. These experiments were puzzling at the time since the "delayed hypersensitive" reactivity was transient and appeared early after administration of antigen, having more the characteristics of Mitchison's "tissue immunity" than classical tuberculin-type delayed hypersensitivity; however, its appearance, its persistence for 48 hours and its occurrence in the absence of demonstrable antibodies, distinguished this reaction from the hypersensitive reactions of the immediate type. Despite the similarities to classical DTH these workers introduced the term "Jones-Mote hypersensitivity" to denote the early transient reaction. They did no histological studies on this skin reactivity. Salvin (1958) was interested in the kinetics of induction of various immune reactivities and noted three distinct temporal phases of response: i) a latent period following antigen injection during which time neither sensitivity nor circulating antibody could be detected; ii) a period of delayed sensitivity during which time no antibody could be detected and which was transferrable only by lymph node cells; and iii) the appearance of circulating antibodies and Arthus type reactivity. The latter was described at this time as "soggy edema" associated with serum precipitins. In 1961, Weigle demonstrated that Arthus reactivity depended on the fixation of complement by antigen-antibody complexes. Salvin (1958) also noticed that increasing the sensitising dose decreased the duration of the delayed type reaction, while a decrease in the dose prolonged the delayed type reactions with a delay in the onset of the Arthus type reactivity.

While it was still not clear whether the stimulation of DTH and antibody were different processes, it became clear that these responses were due to the reactivity of different cells. Selectively removing the avian bursa

(Szenberg and Warner, 1962), and thus the B cells, from immunologically immature animals affected antibody production more than cell-mediated responses; while removing the thymus (Miller, 1961), and thus preventing the generation of T cells, affected cell-mediated responses more than antibody responses (Cooper, Peterson, South and Good, 1966). It was also clear that most antigens capable of inducing antibody responses could, under appropriate conditions, also induce delayed hypersensitivity (Pearson and Raffel, 1971). It was emphasised by these workers that those antigens which were "not very foreign", either because of their small size or their similarity to self antigens, were more likely to induce DTH reactions; while antigens which were very foreign were more likely to induce humoral antibody. This relegated the selective induction of immune reactivities to the immunoregulatory capacity of the responding animal rather than absolute differences between antigens. The question became (and still is): what exactly do T and B cells recognise and why do certain responses prevail after particular immunisation protocols?

2. Subsets and specificity of immunocompetent cells

Before continuing a discussion of the similarities and differences between T cell and B cell induction, it is necessary to point out that it is not yet clear exactly what is recognised by these two cell populations. B cells carry antigen-specific receptors on their surface and, once induced, secrete antigen-specific antibody molecules with an antigen-binding variable portion exactly identical to that found on the cell surface receptor (Warner, 1974), and a constant portion which is one of a small set of class-specific sequences, each responsible for mediating a particular effector function of that molecule. Secreted products have been produced in large quantities from myeloma tumours or normal spleen cells under laboratory conditions and have been studied biochemically and genetically. One cell usually produces antibody of only one specificity, which depends on the variable regions of the light and heavy chains of the antibody molecule. One light chain constant region (kappa or lambda) is expressed per cell with a variable (light) portion attached, and usually only one or two classes or subclasses of heavy chain constant regions (IgM, IgG, IgA, IgE, or IgD) may be expressed per cell at a given time with a variable (heavy) portion attached. T cells are responsible for a variety of specific immune functions and the structures of their antigen-specific receptors and secreted products are generally a mystery. There are T cells that provide helper or amplifier or auxiliary activity to various other immune responses, T cells that provide inhibitory activity against various classes of immune responses, and T cells responsible for the manifestations of the cell-mediated DTH and cytotoxic T cell responses. Like B cells, T cells can be made tolerant to antigen (Chiller, Habicht and Weigle, 1970; Taylor, 1968); can contribute to immunological memory (Raff, 1970; Miller and Sprent, 1971); and can bind and be specifically "suicided" or inactivated with radioactively-labelled antigen, even in the precursor stages (Basten, Miller, Warner and Pye, 1971).

Hammerling and McDevitt (1974) separated mouse lymphoid cells into T and B cell populations, incubated these with radioactive-iodine-labelled synthetic protein antigens [(T,G)-A--L] and did autoradiographic studies to see if both cells could bind protein antigens. It was found that B cells could bind 500-15,000 molecules per cell, while T cells could only bind 200-500, indicating a difference in the number of surface antigen receptors between these cell types. At 37°C., the number of T antigen-binding cells increased two- to three-fold, while the number of B cells binding antigen remained the same. The B cell binding of "hot" (T,G)-A--L could be inhibited by excess unlabelled (T,G)-A--L, (H,G)-A--L, or (Phe,G)-A--L, but the T cell binding was inhibitable only by excess unlabelled (T,G)-A--L, indicating a difference in the specificity of binding.

There are also differences in the "hot" antigen specific suicides of B and T cells as reported by Basten, Miller and Abraham (1975). B cells can be killed at 4°C. and their suicide is not inhibited by azide or anti-H-2 Fab (directed against the whole MHC region); while the suicide of T cells requires incubation at 37°C. and is inhibitable by azide and/or anti-H-2 Fab. Thymus cells also require the presence of another cell type during the incubation (provided in a population of anti-theta and complement treated antigen-primed spleen cells) in order to suicide efficiently. This indicated to the authors that H-2 associated determinants were in close proximity to the antigen-binding site on T cells but not B cells, and might even constitute part of the T cell receptor. These experiments also indicated that T cell suicide required active metabolic functions, while B cell suicide did not. The thymus cells may require another cell surface to focus a large concentration of antigen to increase the avidity of binding or they may require the presence of another self antigen on the cell surface which somehow increases the binding affinity between the cell bearing the antigen (probably a macrophage) and the T cell.

There was controversy for some time over the size of the antigen-specific T cell receptor and whether it was as finely discriminating between antigens as the B cell receptor. It had been noted that in order to elicit DTH reactions in guinea pigs, the eliciting antigen had to have not only the same haptenic group as the sensitising antigen, but also the same carrier protein (Salvin and Smith, 1960; Gell and Silverstein, 1962). This was interpreted as evidence that the T cell receptor recognises an area larger than the hapten, i.e. involving a substantial portion of the carrier protein as well. It was noted at the time that antibody produced in the same system could bind to the hapten on any protein. In direct opposition to these reports are two studies (Waterfield, Levy, Kilburn and Teather, 1972; Spitler, Benjamini, Young, Kaplan and Fudenberg, 1970) which indicate that DTH can be elicited by a hapten alone. Studies by other workers (Janeway, Cohen, Ben-Sasson and Paul, 1975) involved immunising guinea pigs with hapten (DNP) coupled to guinea pig albumin, and, although they still found no T cell responses (DTH, T cell proliferation, specific antibody helper function) to DNP coupled to other proteins on challenge, they found a significant response against DNP-guinea pig albumin. It is possible that most of the T cell reactivity is directed against neoantigenic determinants created by the attachment of the hydrophobic haptenic molecules to the guinea pig albumin. Slight chemical modification of the hapten such as varying the number and position of the nitro groups indicated that the T cell could distinguish between the subtle differences in the hapten molecules in a manner similar to that previously shown for B cell receptors (Landsteiner and van der Scheer, 1936). The studies of Hammerling and McDevitt cited above would tend to indicate that the T cell is, if anything, more discriminating than the B cell in its antigen-specific receptor, based on the binding of antigen and the inhibition of binding by related molecules.

Other studies also indicate that the antigen-specific receptor of cytotoxic T cells is equivalent to that of B cells (Vasquez, Neauport-Sautes and Senik, 1980). Alloreactive cytotoxic T cells were induced against a particular haplotype, and adsorptions were performed with various macrophage monolayers possessing certain, but not all, of the H-2 specificities in common with the inducing antigen. Significant, but reduced, residual lytic activity after adsorption was consistently demonstrated, and could be shown to be due to killer cells specific for H-2 public or private determinants not represented on the adsorption monolayers. These observations showed that a large number of cytotoxic T cell clones, both those adsorbed and not, were activated by alloantigens, and the specificity of these clones was for the same public and private antigenic determinants as those defined by antibody studies. Not only were the same determinants apparently recognised, but even the cross-reactions between determinants were the same. This indicates that the requirements for recognition by the receptor sites on B and T cells are equivalent.

The specificity of T cell receptors and whether more than one type of receptor exists on a given T cell has been a matter of some dispute. Cytotoxic T cells show a curious restriction phenomenon in that, if they have been stimulated by a particular virus or hapten on a self cell, they will exclusively kill targets which carry both the same viral or haptenic group and their own histocompatibility antigens, especially H-2 K and/or D antigens (Zinkernagel and Doherty, 1974). A parallel situation is seen in the systemic transfer of sensitised DTH effector T cells to naive animals (Miller, Vadas, Whitelaw and Gamble, 1975a). If the donor animal has been sensitised by contact chemicals, successful transfer can occur only between those sensitised cells and mice which bear the same H-2 K, D or I region antigens. If sensitisation occurs via antigen-pulsed macrophages, I region matching is necessary between the antigen-pulsed cells used for priming and those used for elicitation of the response (Miller, Mottram, Gamble and Vadas, 1978). A similar requirement for I-region matching has also been found in the T cell proliferation assay in which primed guinea pig T cells are restimulated *in vitro* with macrophage-bound antigen (Paul, Shevach, Pickeral, Thomas and Rosenthal, 1977). Studies in which allogeneic reactivity has been carefully removed show that T cells may respond to antigen bound to non-self macrophages when the animals were primed with antigen bound to these same non-self macrophages (Thomas and Shevach, 1977). The same basic phenomenon is true for the restriction seen between helper T cells and B cells, in that once allogeneic reactivity has been very carefully removed, successful collaboration can occur between histoincompatible cells but the I region gene products, while they need not be self, must be those that were present during priming (von Boehmer and Sprent, 1976).

More recent studies, in which alloreactive cells are removed, show that T cells and "antigen presenting cells" must be "genetically compatible". T cells and B cells need not be, but rather require the linked recognition of carrier and hapten determinants. (Shih, Matzinger, Swain and Dutton, 1980).

Many immunologists believe as a result of the work described above that DTH or helper T cells recognise a combination of antigen and (self) I region, and that T killer cells and some DTH T cells recognise a combination of antigen and (self) H-2 K or D antigens. This has been postulated to occur either by one T cell receptor recognising both antigen and MHC determinants or two T cell receptors one of which binds antigen and the other one of which binds MHC determinants.

It seems to me that there could be a number of factors confusing this issue. In some instances, particularly in the case of cytotoxic T cells, it could be that the antigen-specific receptor actually recognises a self H-2 K or D molecule which has been altered somehow, e.g., been haptenated, or had viral determinants attached, or been altered specifically by viral enzymes. It is known that cytotoxic T cells can be generated against cells carrying only a very slight mutation in the H-2 K antigen molecule (Klein, Forman, Hauptfeld and Egorov, 1975). If the specific antigen is a modified self antigen, it would not be surprising that the killer cells generated were non-cross-reactive with other H-2 antigens plus that hapten or virus. There may be other instances where some H-2 product actually forms a particularly dominant part of an antigen due to some molecular interaction between the antigen and the H-2 product (self or other). The very existence of exceptions to the recognition of antigen plus a particular H-2 K or D determinant for cytotoxic T cells, e.g., those raised against I region determinants (Wagner, Gotze, Ptschelinzew and Rollinghoff, 1975); or F-9 antigens (Wagner, Starzinski-Powitz, Rollinghoff, Golstein and Jakob, 1978), shows that recognition of these H-2 products is not essential for cytotoxic activity. Cytotoxic T cells probably do not have to recognise self H-2 products on the targets, as some of the best studied killer cells are directed against alloantigens. This argument remains inconclusive as certain killer cells specific for alloantigens have been shown to cross-react with altered-self (e.g.,Bevan, 1977).

Another confusing factor is that H-2 restriction may not be due to a positive preference for self H-2 molecules to be present, but to a negative or interfering reaction to allogeneic H-2 molecules being present. Thus, very careful removal of negative allogeneic effects allows the cooperation of most fully allogeneic T and B cells, even when these are already primed (Swain, Trefts, Tse and Dutton, 1976), while the addition of very few allogeneic cells inhibits the cooperation of even fully syngeneic T and B cells (Swain, Trefts, Tse and Dutton, 1976; Waldmann, 1977). One is reminded of the background anti-allogeneic DTH responses found in guinea pigs by Brent and Medawar (1963) and wonders what role pre-existing, non-deliberately-primed alloreactivity really plays in H-2 restriction phenomena. This question will probably not be resolved until the T cell receptor(s) has (have) been isolated and fully characterised biochemically and genetically.

The T cell receptor that is responsible for antigen recognition has been shown to share idiotypic markers with those on the variable heavy chain region of B cell receptors recognising the same antigen, and T as well as B cell immunity can be induced by anti-idiotypic antibody (Binz and Wigzell, 1975; Eichmann and Rajewsky, 1975). Although light chain expression (variable or constant portions) has not been convincingly demonstrated on the surface of T cells, it would seem efficient for the immune system to use the same antigen specific receptors throughout, especially when one considers the elaborate genetic mechanisms evolved to generate those receptors on B cells.

Since much of the knowledge about B cell receptors came from secreted products, several investigators have concentrated on antigen-specific products secreted by T cells to gain information about how T cells recognise antigen. Analysis of these helper factors (Taussig, Munro, Campbell, David and Staines, 1975) and T suppressor factors (Tada, Taniguchi and David, 1976) has not revealed any determinants cross-reactive with the constant portion of the immunoglobulin chains, but they each possess determinants coded by the I region of the MHC. Attempts to detect an I coded portion of the T cell receptor itself have so far been unsuccessful, but by analogy to B cells one might suggest this possibility. It is tempting to think of at least part of the various I regions as coding for different "constant" portions of T cell products and determining their effector function. Again, we must await the results of further biochemical studies on the T cell receptor(s).

Whatever the T cell receptor looks like, there is evidence that it is involved in the control of nearly all immune responses studied, being involved in a cooperative role for the induction of responses and an inhibitory role in the control of other responses. These concepts are reviewed in the following section.

3. Evidence for cellular cooperation for induction of immune responses.

The first system reported demonstrating cellular collaboration for the induction of an immune response was in 1966 by Claman, Chaperon and Triplett. Unprimed syngeneic thymocytes or bone marrow cells were transferred alone or together into irradiated recipient mice which were subsequently primed with antigen (SRBC). Neither cell population alone could produce antibody-forming cells after 5 days, but the two populations synergised to mount an antibody response. Spleen cells adoptively transferred in similar fashion were able to respond to SRBC by themselves. These results were repeated in 1968 by Mitchell and Miller in an attempt to determine which cell source provided the antibody-forming cells and which cell source provided the auxiliary or helper cells. They showed that thoracic duct lymphoid cells could synergise with syngeneic bone marrow (although they formed many plaque-forming cells by themselves), but semiallogeneic thoracic duct cells would not synergise in this system with bone marrow cells syngeneic to the recipient. When semiallogeneic thoracic duct cells and antigen (SRBC) were injected into thymectomised, irradiated, bone-marrow-protected mice, there was synergy in the production of anti-sheep hemolysin (antibody), and anti-H-2 serum treatment revealed that the antibody-forming cells were host derived, i.e., bone marrow cells. The source of the antibody forming cells in an adoptive transfer system in which there was synergy in the induction of a humoral response between bone marrow and thymocytes was determined in a subsequent paper by these same authors (Nossal, Cunningham, Mitchell and Miller, 1968) in which they used fully histocompatible CBA and CBA/T6T6 cells for cooperation experiments. The T6T6 mouse carries a distinctive chromosomal marker and chromosomal analysis of individual antibody forming cells showed them to be derived from the bone marrow and not the thymus population.

Another approach to cellular cooperation is exemplified by the studies of Rajewsky, Schirrmacher, Nase and Jerne (1969), in which the secondary antihapten antibody response was examined in rabbits. They concluded that more than one antigenic determinant is required in order for a molecule to be immunogenic, even for a secondary response. Thus haptens are only immunogenic when coupled to a carrier, and secondary stimulation requires recognition of both hapten and carrier, i.e., the animal must have been previously primed to both parts of the antigen in order to effect a true secondary antibody response. Similar studies in mice were reported by Mitchison (1967,1971), and in this system it was emphasised that, for a secondary challenge, in order to obtain an enhanced secondary response, the hapten used to prime the antibody forming cells must be physically linked to the carrier used to prime the helper T cells. It was insufficient in this system to have both antigens present but not physically linked to one another.

The kinetics of the appearance of a helper population, following in vivo antigen priming, was examined using an in vitro culture of mouse spleen cells (Kettman and Dutton, 1971) and effective helper cells were found to be present to some degree even one day after intravenous (iv) priming of a mouse with approximately 2 x 10^7 heterologous erythrocytes. The helper population was treated with 1000R of gamma irradiation before being added to the culture system to prevent the production of antibody-forming cells from this population, and the helper effect was shown to be resistant to this exposure. The activity of the helper cells peaked around day 3 but was still present 14 days after priming. This helper effect was antigen-specific and again it was shown that a hapten-specific response was only enhanced when that hapten was physically linked in culture to the antigen used to prime the helper population. When T cells were primed by incubating thymocytes and heterologous erythrocytes in a lethally irradiated mouse for 7 days, the subsequently irradiated helper cells were found to be "less specific" than those generated in a normal immunised mouse (Vann and Kettman, 1972). Thus, T cells primed for 7 days to SRBC in an irradiated mouse helped generate a good antibody response to SRBC and a slightly enhanced response to BRBC or HRBC when those antigens were also present in the cultures. HRBC or BRBC educated T cells from irradiated mice only slightly increase the SRBC response *in vitro* when both specific antigen and SRBC are present. This was shown to be due to cross-reactive T rather than B cells since no clear plaques were obtained by B cells incubated with mixtures of the two red cell antigens. This type of observation has been used to argue that T cells are less antigen-specific than B cells (Hoffmann and Kappler, 1973). It must be remembered that the above-mentioned helper T cells generated in the normal immunised mice were not cross-reactive against different heterologous erythrocytes. It may be that more helper T cells are generated in the irradiated mouse and perhaps even those with low affinity for the immunising RBC (some of which have higher affinity for another RBC) are able to be triggered in this system. It has also been shown that T cells require less antigen to be stimulated than B cells

(Parish, 1971; Falkoff and Kettman, 1972), so it may be that a broader range of affinities are induced rather than that those which are induced are more antigen-cross-reactive than their B cell counterparts.

Cellular cooperation (T-B) has also been shown for the induction (secondary) of IgE antibody responses. An adoptive transfer system showed the theta-bearing (T) cells from alum-precipitated Ascaris-primed mice could cooperate with spleen cells from mice primed with DNP-KLH (in alum) to produce a secondary IgE or IgG antibody response in an irradiated host when DNP-Ascaris is provided as antigen. Some secondary IgE could be generated in this system if the antigenic challenge consisted of hapten and carrier on separate molecules (unlinked recognition) and may be a consequence of using adjuvant in the priming protocol, or may be due to an IgE non-antigen-specific potentiating factor from Ascaris (Hamaoka, Katz and Benacerraf, 1973).

The dependence of IgA production on the presence of functional T cells has been shown in neonatally thymectomised rabbits (Clough, Mims and Strober, 1971). The anti-hapten serum IgA response in these animals following challenge with arsanilic acid - bovine serum albumin was more markedly reduced as compared to normal rabbits than was their IgM or IgG response. These observations led the authors to propose that, at least for certain antigens, the serum IgA antibody response is more T dependent than other immunoglobulin classes such as IgM or IgG.

Cooperation between two sets of T cells bearing different antigenic markers has been reported in the production of cytotoxic T cells. Cantor and Boyse (1975) showed that peripheral T cells bearing Ly23 antigens could be amplified or helped to develop alloresponsive killer activity by T cells bearing Ly1 antigen. A system involving the maturation of alloantigen-specific T killer cells from thymocyte precursor cells *in vitro* showed a requirement for the helper T cells to be antigen-specific (Pilarski, 1977). Thymocytes incubated with irradiated allogeneic spleen stimulator cells (antigen) would not support the generation of killer T cells, but did so when irradiated normal spleen cells syngeneic to the thymocytes were added. By using spleen cells and thymocytes from two mouse strains that carry the same major histocompatibility antigens but differ in their Thy-1 alleles, i.e., CBA/CaJ and AKR/J, it was possible to determine which population of cells gave rise to the killer effector cells. The effectors came from the thymocyte population and required the presence of theta-bearing (T) spleen cells which were not tolerant of the alloantigen used to stimulate the cytotoxic T cell response. CBA spleen cells made tolerant of Balb/c antigens by reconstituting an irradiated Balb/c mouse with CBA bone marrow cells, could help induce an anti-C57Bl/6 but not an anti-Balb/c cytotoxic T cell response when incubated with irradiated spleen (antigen) and CBA thymocytes. This is another example of fully differentiated (radioresistant) anti-allogeneic activity being present in normal lymphoid cells obtained from an animal that had not been deliberately primed, and, as indicated previously, this alloreactivity may be relevant to the phenomena associated with H-2 restriction. The physical linkage of a determinant to which the animal is tolerant (Balb/c) and a target determinant, using (Balb/c x C57Bl/6)F_1 targets, did not interfere with the generation of killer cells, making the participation of suppressor or inhibitory T cells most unlikely in this system.

T-T cooperation has even been shown in the generation of helper T effector cells which allow the *in vitro* production of antibody (Feldmann, Kilburn and Levy, 1975). This system involves the use of an antigen (performic acid oxidised ferrodoxin, OFd) which has essentially only two antigenic determinants, one at the COOH-terminus (C-hapten) and one at the NH2-terminus (N-hapten). Mice were primed to either hapten separately, by administering hapten-BSA conjugates, and it was shown that a culture of cells incubated with either hapten would not induce help for a subsequent anti-DNP-OFd antibody response. A mixture of the two separately primed spleen cells or cells primed to the whole molecule, OFd, could induce help in culture for a DNP antibody response on subsequent incubation with DNP-OFd. The induction of help was enriched by T cell enrichment and was sensitive to anti-theta plus complement treatment. The cooperative response leading to the induction of help could be inhibited by excess hapten-BSA in an antigen-specific fashion, and specific suicide of either hapten-specific population by radioactive antigen resulted in the loss of the induction of the OFd helper response. These results indicate that cooperation between two antigen specific helper T cells is necessary in order to generate _in vitro_ helper effector cells that can cooperate in an anti-DNP-OFd antibody response.

T-T collaboration has also been shown for the induction of "suppressor" or inhibitory T cells. Feldmann and Kontiainen (1976) induced T cells _in vitro_ which could inhibit specific antibody production. The cells were incubated with KLH at 100ug/ml, or 1000 times the optimal concentration for inducing anti-KLH antibody, and these cells were able to specifically inhibit the induction of the anti-hapten response obtained by challenging with the hapten coupled to KLH. They were shown to be T cells and to require collaboration between two populations of anti-B cell treated spleen cell populations to be generated. One population of T cells was obtained from adult thymectomised mice, and gave rise to the inhibitory T cells. The necessary collaborating population consisted of those splenic T cells that had survived in mice treated with anti-lymphocyte serum (ALS). In this case, it is not known whether the T-T collaboration is specific. Another report claims to show T-T collaboration in the generation of those T cells able to inhibit contact sensitivity (Sy, Miller, Moorhead and Claman, 1979). These workers generate T cells that inhibit the activity of DTH T cells expressing contact sensitivity to DNFB generated in normal mice when they are both transferred to a naive recipient. If the DTH effector cells come from cyclophosphamide-treated mice, these T cells do not inhibit the transfer of contact sensitivity. The authors assume the cyclophosphamide pretreated mice are lacking a drug-sensitive auxiliary T cell necessary for the induction of the inhibitory cell, and conclude that T-T collaboration is probably essential for the generation of these inhibitory cells.

T-T cooperation for the induction of DTH has never been demonstrated as far as I can determine. In 1975, a report was published which claimed to demonstrate helper cells for the induction of this class of immunity, but the data on which the argument was based were not presented in the publication as described, and so cannot be adequately assessed (Bullock, Katz and Benacerraf, 1975). Other workers in the DTH field report a "lack of evidence for helper T cells in DTH" induction (Smith and Miller, 1979). These experiments involve DTH induction against alloantigens and, as generally believed and outlined previously, the helper T cells preferentially recognise I region differences between strains while other effector T cells, e.g., cytotoxic T cells and some DTH effector T cells, preferentially recognise K or D region differences. The magnitude of the DTH response induced in this system was equivalent whether it was raised against cells which differ only in the K region of the MHC or in both the K and I regions. It was concluded from these observations that there was probably no helper T cell involved in the alloantigen DTH response. This conclusion assumes that any putative helper T cell required for the induction of DTH is limiting the size of the DTH response when there is only a K-region difference. The study to be described in this thesis will demonstrate that T-T interaction between populations of cells is necessary for the induction of DTH to a xenogeneic red cell antigen.

Arguments have been presented suggesting that the cell population responsible for helper activity (in antibody induction) may be the same as that which mediates footpad swelling reactions (Kettman,1972). The two populations were compared on the basis of the kinetics of sensitisation, the dose of antigen required for sensitisation (too small to elicit a humoral response), the antigen specificity and cross-reactions of the induced population(s), and induction following injection of specific antisera with a large dose of antigen. In all cases, the properties of the cell population(s) responsible for the two functions were very similar. It was suggested that they might be mediated by the same population of cells. It has been noted by other authors (e.g. Coe and Salvin, 1964; Kappler, Hoffmann and Dutton, 1971) that animals having experienced a DTH response to a given antigen, may appear to be primed for a secondary humoral response on reexposure to that antigen. It is very difficult to determine which population of specific cells is responsible for these functions in situations where mixed populations of antigen-reactive cells are present, such as the whole animal. A very recent preliminary report has been published (Bianchi, Hooijkaas, Benner, Tees, Nordin and Schreier, 1981) in which an antigen-specific "clone of helper T cells" was also shown to result in a specific increase in footpad thickness when injected with antigen. It may be that the same antigen-specific precursor T cells differentiate into different populations to give these different activities, or, that the potential for all antigen-specific T cell functions exists within all of these cloned cells, and they respond according to the signals they receive in their particular microenvironment at a given time. *In vivo*, the expression of these activities (in the absence of adjuvant priming) appears to be regulated such that when a DTH response to a given antigen dominates, there is no antibody response, and vice versa (Parish, 1971, 1972b; Ramshaw, Bretscher and Parish, 1976,1977). If the expression of an antibody response requires helper T cells which are equivalent to DTH effector T cells, the regulation would have to occur at the level of the biochemical mediators of these activities. Purification of the biochemical mediators of these various activities should lead to the development of techniques which will allow one to test which functions a particular cell can mediate in a given state.

4. Immunoregulation and "immune deviation"

i) Self non-self discrimination.

Events determining whether or not an immune response is to be induced against a given antigen, and what class of immune response it should be if there is to be one, have been a puzzle to scientists since immunity was discovered. The ability of the higher animals to respond to nearly every foreign antigen and yet, in most instances, inflict minimal damage on their own self components which are made of the same basic chemical structures demands fine specificity and regulation of the response. The very existence of autoimmunity reminds one that individuals have the genetic capacity to respond to self components and that this must be strictly controlled. The clonal selection theory (Burnet, 1959) hypothesised that individual cells react against individual antigenic determinants, so the control of autoimmunity might be the elimination of particular cells which are capable of recognising and reacting against self antigens. If this occurs at the level of newly generated thymocytes it could account for the discrepancy between the very large number of thymocytes generated daily and the number of functional immunocompetent thymus-derived cells leaving that organ daily (Nossal, 1964; Murray and Woods, 1964).

Attempts to discover what is distinct about one's self components led to the conclusion that most major antigens are present in the animal before the immune system develops and remain present throughout the entire life of the animal. Numerous experiments have now been performed which indicate that the body learns what is "self" by that antigen's continuous presence. Thus, if a self component is selectively removed from an animal for some time and then returned, it is immunologically rejected. Some examples are the buccal component of the hypophysis of the tree frog *Hyla regilla* (Triplett, 1962); the Bursa of Fabricius in chickens which causes the immunological rejection of transferred syngeneic B cells but not T cells when these are injected (Grebeneau, Lerman, Paladino and Thorbecke, 1976); and the C5 complement component in C5 deficient mice (Allison, 1971). On the other hand, "foreign" self-replicating antigens introduced to an animal at an early stage of ontogeny, e.g., fetal stem cells in certain cattle twins (Owen, 1945) or Avian Leukosis Virus in chickens (Rubin, Fanshier, Cornelius and Hughes, 1962) are accepted as "self" antigens and no detectable immune response is raised against these. An attempt to explain how such a self-learning mechanism could account for clonal elimination of anti-self cells led to Lederberg's (1959) proposal that all newly generated immune precursor cells, before they differentiate to an inducible state, pass through a phase during which they will be paralysed if they encounter specific antigen. This is still a popular idea with some immunologists and attempts to examine these ideas experimentally are in progress in various laboratories (Nossal and Pike, 1978) This theory does not account for the generation of autoimmunity when it does occur, and the mechanisms postulated could be detrimental during chronic infections. The Bretscher-Cohn hypothesis (1970) assumes that the outcome of a meeting between an antigen and an antigen-specific precursor cell will be determined by the presence or absence of a second cell able to recognise a second determinant on the same antigenic molecule. If the precursor cell meets the antigen in the absence of an antigen-specific T cell, the precursor cell will be paralysed ("one signal only"). Thus self tolerance against self antigens present at reasonably high concentrations could result from the deletion of the individual precursor cells specific for those antigens, as they are generated one by one, and encounter the self antigen in the absence of any cooperating cell, and are thus paralysed. Persistence of immunocompetent cells specific for self antigens present at lower concentrations, or with low affinity for self antigens present in larger amounts, could conceivably occur according to this scheme and autoimmunity could be induced. Such autoimmunity is likely to be induced against those self components which have foreign determinants physically linked to them, e.g., during a viral infection. The presence of immunocompetent cells specific for foreign determinants would then depend on the semi- random generation of receptor specificity and accumulation of all those cells that do not have high affinity for common self determinants. All other specificities should encounter "helper" cells for the induction of immune responses when they meet an antigen with sufficient foreign sites ("two signals": one from antigen, one from an antigen-specific cooperating T cell).

Since not all anti-self precursor cells are eliminated, (indeed, considering the variety of self components, if every cell that could have any affinity for any self determinant were paralysed, the immune repertoire would be severely depleted) it is likely that there are other controls on the immune system that minimise the effects of autoimmunity. It has been postulated that the controls in question are responsible for the phenomena of "immune deviation" and "split tolerance" observed in various experimental situations (Asherson, 1967; Bretscher, 1974; Bretscher, 1977).

ii) Immune deviation or split tolerance.

This basic phenomenon can best be described as an antigen-mediated and antigen-specific depression of certain classes of immune response while leaving another class of immune response intact. The term "immune deviation" was originally proposed to describe an observation by Asherson and Stone (1965) in which the delayed skin reaction usually induced in guinea pigs immunised with antigen in complete Freund's adjuvant was reduced by prior treatment of the animals with soluble or alum-precipitated antigen. Little antigen was required in the original or deviating dose, and it could be administered intravenously or subcutaneously in the footpad 14, 7 or 1 day before or 1 day after the challenge with antigen in adjuvant. The reaction is antigen specific in that prior treatment of guinea pigs with soluble or alum-precipitated bovine gamma globulin, as opposed to human serum albumin, did not result in a reduced DTH reactivity to human serum albumin. The antigen specificity of this phenomenon was again demonstrated by Crówle and Hu (1966) using the antigens ovalbumin and bovine serum albumin in essentially the same protocol as Asherson and Stone, with the same observations, and they termed the phenomenon "split tolerance". It was also demonstrated that guinea pigs rendered serologically unresponsive (i.e., no antibody production) to PPD by neonatal injection were fully capable of demonstrating a hypersensitivity to PPD (Janicki, Schecter and Schultz, 1970).

In more controlled experiments, in which adjuvants were not used, mice injected with HRBC and expressing humoral immunity for that antigen were shown to contain antigen-specific T cells which actively inhibited the induction of antigen-specific DTH. This was demonstrated by transferring these cells to cyclophosphamide-treated mice subsequently immunised to develop a DTH reaction to that antigen (Ramshaw, Bretscher and Parish, 1976). It could also be shown in this system, by inducing a protein-specific humoral response, that mice mounting an antibody response could inhibit the induction of DTH to HRBC determinants physically linked to that protein. It was also demonstrated that mice treated with cyclophosphamide and immunised with HRBC to develop strong delayed hypersensitivity to that antigen not only displayed no humoral immunity to the antigen but also contained T cells which would specifically inhibit the induction of a humoral response by normal spleen cells in an adoptive transfer system (Ramshaw, Bretscher and Parish, 1977). This induction of antigen-specific immunity in one class of response with concomitant induction of antigen-specific inhibitory T cells of another class of immunity may well be the cause for the inverse relationship between the induction of humoral and cell-mediated immunity observed when investigators use the same antigen in the native state or chemically modified by acetoacetylation. This procedure has been shown to reduce the immunogenicity of a compound without grossly altering the specificity of the remaining antigenic determinants, i.e., they still bind antibody raised against the native antigen. While native antigen, flagellin from *Salmonella adelaide* (Parish, 1971) or SRBC (Parish, 1972a), normally induces humoral immunity when injected into rats ip or sc, increasing degrees of acetoacetylation of the antigen reduced the humoral response while enhancing the cell mediated response. A similar phenomenon was shown with CNBr fragments of flagellin versus the native antigen. Even when both types of response are induced following immunisation with antigen in adjuvant, they tend to dominate the response sequentially as mentioned earlier in the experiments by Salvin (1958).

The inverse relationship between the induction of DTH and antibody-forming cells can also be seen *in vitro* (Ramshaw and Eidinger, 1979; Bretscher, manuscript in preparation). A constant number of normal spleen cells cultured with various concentrations of antigen (HRBC) mount only a DTH response at low concentrations, only PFC at medium concentrations, and only DTH at the highest concentrations (Ramshaw and Eidinger, 1979). The same basic phenomenon is demonstrated when the antigen concentration is held constant but the number of normal spleen cells is increased either in cultures (Bretscher, manuscript in preparation) or in adoptive transfer systems (Bretscher, manuscript submitted). Low numbers of normal spleen cells mount DTH reactivity against an antigen while higher numbers of spleen cells with the same antigen concentration induce a PFC response.

Mouse spleen cells can also be induced to generate preferentially IgE or IgG anti-hapten production *in vitro* by mice that have been primed with low (0.01ug) or higher (10ug) doses of Ascaris respectively, indicating that the conditions of priming, by changing only the antigen dose, favour the induction of a particular class of immunity (Kimoto, Kishimoto, Noguchi, Watanabe and Yamamura, 1977).

It has long been observed that feeding of a particular antigen to animals renders them imunologically anergic to that antigen (Wells, 1911). Chase (1946) fed allergenic compounds to guinea pigs and subsequently tried to skin sensitise those animals with the same compound. In a well controlled experiment, all control animals developed contact sensitivity to the substance 2:4 dinitrochlorobenzene while the experimental animals demonstrated antigen-specific resistance to skin sensitisation. "Tolerance" to systemic DTH reactions by prior feeding of haptens became known as the Chase-Sulzberger phenomenon and was later associated with the induction of T cells which specifically suppressed the induction of DTH, IgM, IgG (Mattingly and Waksman, 1978) and IgE (Ngan and Kind, 1978); and with the induction of antigen-specific IgA (Challacombe and Tomasi, 1980). The phenomena described following intragastric administration of antigen can only be induced against those antigens to which an animal is not already immune; in this situation, oral administration of antigen tends to immunise systemically rather than "tolerise". It has not been determined whether IgA was produced to the antigen against which the animal is already systemically immune.

The mechanism(s) by which cellular cooperation functions in immunoregulation is still a matter of some controversy. Helper T cells recognising an antigen and cooperating with another cell for induction must ensure that the right (i.e. antigen-specific) cell is triggered. This may occur by helper T cells specific for antigen, which induce other immunocompetent cells recognising the same antigenic molecule; or by helper T cells which recognise idiotypes on the antigen-specific receptors of immunocompetent cells. It has been possible to demonstrate regulatory T cells, both positive (Woodland and Cantor, 1978) and negative (Ward, Cantor and Nisonoff, 1978), specific for the idiotypes present on certain "dominant clones" in various mouse strains. These dominant clones are cells with particular idiotypes that represent a substantial proportion of the cells responding to a particular antigen, typically a bacterial carbohydrate. It is not yet known whether this idiotype-specific regulation is a general mechanism, or if it operates only to regulate dominant clones. Studies demonstrating a requirement for linked recognition show a need for short range inductive signals between cells specific for the antigen, whether or not idiotypic determinants are also recognised.

In summary, there are numerous experimental systems in which one class of immunity is induced and, at the same time, other classes against the same antigen, or determinants physically linked to it, are actively inhibited. Moreover, there exists a type of hierarchy of response, at least as far as CMI, IgM and IgG (or IgE and IgG) are concerned. For a given number of responding cells, IgG requires more antigenic stimulation, IgM less and CMI the least; or IgG requires the most responding cells, IgM less and CMI the least if the antigen concentration is held constant. Careful quantitation of factors involved in the induction of these various classes suggests that the hierarchy depends on the number of functional triple complexes of antigen, antigen-specific precursor cell and antigen-specific helper cell (or helper factor) that are available during the inductive phase. This hierarchy of response for DTH, IgM and IgG, and its dependence on the number of functional triple complexes present was predicted on theoretical grounds (Bretscher, 1974,1977). Experiments designed to test the assumptions made in the theory of immune class regulation have so far been consistent with the theory, including those presented in this thesis.

iii) Biological significance of "immune deviation"

It has been known for a long time, largely through "experiments of nature", that various classes of immune response are most effective against different pathogens. CMI is necessary to fight off viral and fungal infections as well as certain bacterial infections, particularly against those organisms that tend to live intracellularly (e.g., Brucella, Salmonella, Mycobacteria, etc.). Humoral immunity is essential to protect against other bacterial pathogens as evidenced by the repeated bacterial infections suffered by agammaglobulinemic individuals (Cooper and Seligmann, 1977). Patients deficient in IgA production tend to have higher instances of allergies or immediate hypersensitivity reactions and a higher incidence of autoimmunity (Tomasi,1980). Moreover, those who are not immunodeficient but produce the wrong class of immunity against a particular organism (e.g., humoral antibody against Mycobacterium tuberculosis or Mycobacterium leprae) suffer from more severe disease than those individuals producing an appropriate class (Paul, 1980). Obviously, some classes of response deal more effectively with certain foreign invaders than other classes. This pattern can be seen to correlate with the hierarchy of responses previously described. A virally infected cell contains many self determinants with relatively few foreign ones, and the number of triple complexes formed early in a viral infection will therefore be small, favouring the induction of cell-mediated immunity. As the virus multiplies, the effective antigen concentration increases and more complexes will be formed, favouring progression of the response into humoral immunity (IgM, then IgG). This is exactly the sequence seen following *Paramyxovirus* infections of mice. Furthermore, the expression of viral specific cytotoxic T cells and IgG against *Sendai* virus infections of C57Bl/6J mice are mutually exclusive (Tucker and Stewart, unpublished observations). A bacterium carries more foreign determinants and most (with the exception of those that tend to live intracellularly mentioned earlier) tend to induce humoral immunity if they penetrate the mechanical barriers of the organism to produce an infection. The resident mucous membrane flora induce predominantly IgA reactivity, which protects the individual from invasion, but does not destroy these necessary bacterial commensals. Even IgE appears to be beneficial, especially in the response against helminthic parasites (Ogilvie and Love, 1974), although the evidence is not yet completely convincing. There are conflicting reports about the health of IgE deficient human patients

(Ammann, Cain and Ishizaka, 1969; Levy and Chen, 1970), so the benefits of this class remain somewhat doubtful. One should not assume a response is only harmful beacause one sees only the harmful effects induced by that particular class. DTH used to be thought of as a detrimental type of immune response because the lung damage seen in TB patients or the granuloma formation seen in patients with tubercle-type leprosy is largely due to the DTH reactivity associated with these infections rather than the action of the offensive organism itself. This is true to some extent, yet if an antibody response is raised against Mycobacteria it is completely ineffective at controlling the organism, and the result is lepromatous leprosy or miliary tuberculosis.

Taken together, these ideas indicate that particular immune responses are beneficial in various different situations, while inappropriate responses may actually be detrimental. If there were no class exclusiveness and all responses were induced concomitantly against all antigens, they might interfere with each other's efficacy (e.g., enhancing antibody protects tumours from CMI destruction), so there is good biological sense in having only one class of immune response at one time. This should be the type of response most effective against that particular antigen.

The more "foreign" the antigen is, the more specific precursor T cells there will be recognising this antigen, the more triple complexes of antigen plus antigen-reactive cells there will be, and the more the response will tend to humoral immunity versus cell-mediated immunity.

Extensive arguments have been presented by Bretscher (1974, 1977) indicating how this scheme might minimise autoimmune effects of induced anti-self reactivity. From the point of view of this study, it is relevant that this theory postulates specific cellular cooperation to be necessary for the induction of all classes of immunity. To date, cellular cooperation has been demonstrated for the induction of nearly all classes with the exception of DTH.

Before presenting evidence for my demonstration of T-T cooperation in the induction of DTH, I should like to review the various systems recently employed to induce DTH reactivity. I shall emphasise those studies in which mouse cells are induced to mount DTH responses against xenogeneic erythrocytes.

5. The DTH reaction

Early attempts to dissect the DTH response focussed on the elicitation of the reaction. Many early experiments were done in guinea pigs and the reactions were estimated by eye or the diameter (mm) of "redness" around an injection site of depiliated skin. Nelson and Mildenhall (1967) were among the first to introduce footpad thickness after local antigen injection into sensitised mice as a reliable method of measuring DTH, although it remains slightly subjective. These workers also correlated the induction of DTH to SRBC with the production of a "globulin component" in the serum of sensitised mice which was cytophilic for peritoneal exudate cells (PEC, presumably macrophages) from unimmunised mice. Following incubation of the serum with normal PEC, the SRBC could be specifically rosetted by cells incubated with serum from SRBC-immunised as opposed to unimmunised animals. This cytophilic "antibody" component was seen only at a time when DTH could also be elicited in the footpad and before the appearance of SRBC hemagglutinating or complement-fixing antibodies in the serum. This idea of specific T cell factors being transported on the surface of another cell in which form they can be active could explain

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the apparently-specific role attributed to macrophages in various immune responses. This possibility is extremely appealing as it gives this non-specific but necessary cell a means to participate in immune reactions in an antigen-specific fashion. Specific suppression of contact sensitivity can also be transferred by PEC that resisted anti-theta plus complement treatment but was sensitive to trypsin treatment (Asherson and Zembala, 1974). The generation of this suppressor factor was shown to be sensitive to anti-T cell reagents, was antigen-specific, and was passively transferred by a macrophage.

The swelling caused by the local injection of antigen into an animal actively or passively sensitised for a DTH reaction to that antigen is caused by the influx of mononuclear cells as shown by the studies of Dienes and Schonheit (1932). These cells can be shown by radiolabelling studies to be largely nonspecific cells that have been newly generated and are host-derived (McCluskey, Benacerraf and McCluskey, 1963). When two populations of sensitised cells are generated against two non-cross-reacting antigens (diphtheria toxoid and parachlorobenzoyl chloride), and one of these populations is radioactively labelled before both are transferred to a single animal, an apparently equal, small number of labelled cells appear in distant sites when challenged with either antigen. Labelling the animal itself approximately two days before the sensitised cells are transferred results in abundant label appearing in the challenge site to either antigen. Very few antigen-specific cells then are necessary in the site to cause the massive cellular infiltration characteristic of the DTH reaction.

Various labelling techniques were used to detect DTH reactions after it was discovered that the animal itself could be labelled. This allowed smaller reactions to be detected, especially if one measured the radioactive influx into a swollen pinna of a mouse ear. Sabolovic, Buegnot, Dumont and Bujadoux (1972) managed to demonstrate DTH to SRBC without the use of adjuvant using these types of assay methods. This increase in radioactivity could be shown to be T cell dependent since it did not occur in nude or thymectomised mice to the same extent.

DTH reactions are always characterised by some combination of the following criteria: their delayed onset (12-24 hours), their long duration (3-4 days), their mononuclear cell infiltrate, their T cell dependence, antigen specificity and transferrability by cells as opposed to serum (Cooper, 1972; Cooper and Ada, 1972).

Adjuvants were usually required to induce the responses until recently, and it was often difficult to distinguish true DTH reactions from other skin reactivities unless great care was taken to use an appropriate assay. The discovery of cutaneous basophil hypersensitivity in the guinea pig, which gives a maximum skin reaction at 24 hours, is not necessarily associated with detectable circulating antibodies, and can be passively transferred by cells and not serum (Richerson, Dvorak and Leskowitz, 1970) led some to question what type of reaction was being measured by various assays. The skin reaction induced in cutaneous basophil hypersensitivity is a _flat_ well-circumscribed erythema with little or no induration, is usually gone before 48 hours, and studies with carrageenan have shown that the macrophage is not an important contributor to the reaction. These investigators ventured to say that any skin sensitivity generated, especially those against protein antigens or hapten-protein conjugates in the absence of Freund's complete adjuvant, was probably due to this type of reactivity rather than tuberculin-type reactivity. This is clearly not the case, for a flat erythema would not represent any significant swelling in a footpad measurement assay. It was shown by Lagrange, Mackaness and Miller (1974) that SRBC injected iv or sc in saline without any adjuvant, resulted in a classical DTH reaction and could be measured by specific swelling following the injection of antigen. They found that a rapid transient (day 4-6) DTH state was induced by the iv injection of comparatively low doses of SRBC (10^5), and a slightly slower developing but longer lasting (day 4-9) state of DTH reactivity could be induced by the sc injection of larger (10^8) doses of SRBC. Formal histology on mouse feet following the elicitation of SRBC-specific DTH in mice immunised without adjuvant was reported in 1978 (Mitsuoka, Teramatsu, Baba, Morikawa and Yasuhira, 1978) at which time this reaction was shown to be lacking in basophils and displaying the histology of a classical tuberculin-type hypersensitivity reaction.

One must be careful in the use of radioactivity used to measure DTH reactivity because, depending on which components in the animal have been labelled, an accumulation of radioactivity could be detected at the site of any inflammatory reaction induced. This could occur by the influx of polymorphs, or basophils, or merely fluid accumulation (even simply hyperemia) with accompanying labelled proteins. An increase in thickness should measure only a cellular infiltrate if the anatomical area is swollen due to a true induration rather than mere fluid accumulation. In the case of an Arthus type reaction, the swelling should collapse with application of the calipers used to measure the ear or footpad thickness; and in the case of basophil hypersensitivity, the area is flat and erythematous rather than swollen and indurated as in a true DTH reaction.

DTH reactivity can be measured in various ways. The animal itself may be sensitised to give a DTH reaction and then have antigen injected at a particular site, or an animal may be passively sensitised by systemic transfer of sensitised cells with antigen being injected into the ear or footpad. A local transfer of a set number of sensitised cells with or without antigen into the footpad of a naive mouse can also be used.

A study in 1976 (Robinson and Naysmith) compared 4 methods for measuring cutaneous DTH reactions to protein antigens (KLH and HSA) in mice. After inducing DTH with 1mg. of protein in CFA, the specific skin reactions of these animals were measured by the techniques of: increase in ear thickness, increase in footpad thickness, arrival of ^{51}Cr-labelled syngeneic lymph node cells or [^{125}I]UdR accumulation at the challenge site (ear). It was observed that the most sensitive and reliable test was the increase in ear thickness. Increase in footpad thickness was found to be reliable if strong DTH reactions were being measured.

It was discovered in some studies of systemic transfer of sensitised cells, that irradiated sensitised cells could not transfer sensitivity to normal recipients (Kettman and Mathews, 1975). However, irradiated sensitised cells could transfer sensitivity when given locally to the footpad with antigen. This was not possible if the host animal had been irradiated. These observations suggested that the cells responsible for initiating the reaction were radioresistant themselves, but required a radiosensitive population from the host in order to elicit a reaction. Miller, Vadas, Whitelaw and Gamble (1975b) showed that the systemic transfer of lymph node cells sensitised to give a contact sensitivity reaction to DNFB could give antigen-specific ear swelling in a thymectomised, bone marrow protected recipient, but the transfer was sensitive to anti-theta treatment. It therefore appears that a radioresistant sensitised T cell, when present in the same site as specific antigen, orchestrates an influx of non-specific radiosensitive non-T mononuclear cells into the site during the course of a reaction.

The systemic transfer of cells sensitised for DTH reactivity is subject to allogeneic restriction phenomena as reviewed earlier, but the local transfer of cells and antigen has been shown to be non-restricted (Lubet and Kettman, 1979). There is a requirement for syngeneic adherent cells in addition to the sensitised T DTH cells in the locally transferred DTH reaction for maximum swelling. This has been shown by the depletion of adherent cells from a sensitised population and subsequently injecting the nonadherent cells plus antigen into the footpad of a naive mouse. A strong DTH reaction is elicited only in the case of an H-2 syngeneic mouse in this situation. The addition of normal spleen cells, syngeneic to the sensitised cells, allows the elicitation of DTH in a histoincompatible host, as measured by footpad swelling.

A number of in vitro correlates of DTH have been studied in an attempt to find a reliable, less subjective, and less expensive assay for the measurement of DTH reactivity. These include MIF assays (Feinstone, Beachey and Rytel, 1969); macrophage aggregation assays (Phillips, Carpenter and Merrill, 1972); blastogenic responses usually measured by radioactive thymidine incorporation (Warnatz, Scheifforth and Gollnick, 1972); and antigen-dependent cytotoxicity against innocent bystander cells (Ruddle, 1979). None of these tests is completely consistent with the development of footpad swelling reactions to the exclusion of other classes of immune response (Crowle, 1975) and are therefore questionable techniques for the study of tuberculin-type DTH reactions as defined in the literature.

With the advent of techniques to induce DTH reactivity *in vitro* and measure the reactions by the injection of sensitised cells plus or minus antigen in the footpad of a naive mouse (Bretscher, 1979; Ramshaw and Eidinger, 1979), it became possible to dissect the cellular requirements for the induction of DTH. The study to be presented will show that the induction of DTH reactivity *in vitro* requires the cooperation of a specifically primed T cell, whose effects are radioresistant, and which must be specific for a determinant physically linked to the determinant against which the DTH reactivity is to be directed.

II. MATERIALS AND METHODS

1) Animals.

CBA/CaJ mice, aged 6-12 weeks, were obtained from the University of Alberta animal facility. Male or female mice were used, the sex being kept constant throughout individual experiments. These mice were used for all experiments unless specifically stated otherwise.

AKR/J mice, aged 6-12 weeks, were obtained from the Jackson Laboratories, Bar Harbour, Maine, U.S.A..

2) Antigens.

i) Erythrocyte antigens.

BRBC were obtained from the Colorado Serum Company, Denver, Colorado, U.S.A.. They were stored refrigerated in Alsever's solution and were replaced with fresh cells, purportedly from the same animal, every month. The cells were washed three times with normal saline before use.

CRBC were collected from a particular bird (genotype B^2/B^2 at the major histocompatibility complex of the chicken). Ten ml. were collected into a heparinised syringe, and the cells were washed immediately three times in Leibovitz medium and stored in this medium in the refrigerator for not more than two weeks. The cells were washed three times with normal saline before use.

MRBC were collected into Alsever's solution from CBA/CaJ mice. These cells were washed three times in normal saline and used within two days of collection.

ii) Protein antigens.

FGG was prepared from whole chicken serum collected from a slaughter house. The 33% ammonium sulphate precipitate, pH 7.0, was purified and lyophilised by Drs. Bretscher and Diner according to the method of Miller and Sprent (1971). The protein preparation was collected and judged to be pure FGG by the criterion of immunoelectrophoresis.

Alum-precipitated FGG was prepared by the method of Chase (1967). Equal volumes of 10 mg./ml. protein solution and 10% potassium aluminum sulphate were mixed, and the pH adjusted with sodium hydroxide until maximum cloudiness developed (pH 7.0-7.2). The precipitate was washed several times with normal saline, and dilutions of the precipitate were suspended in 25ul. volumes and injected into the footpads of normal mice. The largest concentration of precipitate that gave no non-specific swelling reaction in the footpad was used as antigen to test for FGG-specific DTH.

iii) Coupled antigens.

For chromic chloride coupling, a 1% chromic chloride ($CrCl_3$) in saline solution was adjusted to pH 5.0 every day until the pH remained stable (approximately three weeks). This was then diluted to 0.01% in saline and added dropwise with continuous agitation to a tube containing 0.1ml. of packed RBC, 0.9ml. of saline and 40ul. of a 10mg./ml. protein solution in saline, until slight haemagglutination could be detected (Gold and Fudenberg, 1967). Coupled cells were then prepared in other tubes using slightly less than haemagglutinating amounts of $CrCl_3$ solution (e.g., 500-600ul. vs 800ul.). The cells were allowed to stand at room temperature for 10 minutes, and the reaction was stopped by the addition of cold PBS. The cells were washed three times with saline containing 5% FCS, and titred by microhaemagglutination with a mouse anti-FGG antibody to determine that the cells had protein on their surface. The amount of $CrCl_3$ required to obtain coupled cells showing the highest hemagglutination titre with anti-FGG antibody was used to couple cells for experiments set up on that particular day.

FGG was also coupled to red cells by specific anti-RBC antibody made by immunising chickens against the target red cells. FGG anti-BRBC was prepared by injecting chickens according to the method of Miller and Warner (1971). Fowl were immunised iv with 2.5 x 10^9 BRBC, boosted at two weeks with 2.5 x 10^9 BRBC iv, and the serum was collected after 10 days. Aliquots of 0.5ml. of this antiserum were stored frozen until use. This antiserum agglutinated 2%BRBC to a dilution of 1:256.

FGG anti-SRBC and FGG anti-MRBC had been prepared by Dr. Bretscher by the same method, except that rat RBC were used to immunise the birds for the anti-MRBC antiserum. This antiserum had a very high titre against MRBC (1:1024), and none against BRBC.

FGGBRBC conjugates of this type, when used as antigen, were prepared by incubating the red cells with a 2-fold lower amount of FGG-anti-RBC antibody than that required for hemagglutination for 30-60 minutes in a 37°C. waterbath. The cells were then washed three times with normal saline before use in culture or for footpad injections.

3) Immunisation.

Mice were primed iv with 0.5mg. FGG dissolved in 0.2ml. of physiological saline.

Mice primed iv with RBC were given the appropriate number of cells suspended in saline in a volume of 0.2ml..

The lateral tail veins were used for these injections.

Mice primed sc with RBC were given erythrocytes in saline in a total volume of 25ul. per hind footpad.

4) Preparation of cell suspensions and culture conditions.

Single spleen cell suspensions were prepared in Leibovitz medium by mincing the spleen cells and gently passing them through a stainless steel wire mesh. After the cell clumps had settled for 2 minutes on ice, the cells suspended in the supernatant were removed, washed twice in Leibovitz medium, and the viable cells were counted by trypan blue exclusion. Those cells requiring irradiation were exposed as single cell suspensions to a ^{137}Cs source (Gamma Cell 40, Atomic Energy of Canada Ltd.) for the time required to receive 1500R (approximately 15.9 minutes). The cells were again washed before being added to the cultures.

The culture medium (MEM) consisted of Eagle's minimum essential medium supplemented with 10% FCS (Grand Island Biological, N.Y.,U.S.A.), 10^{-4} M. 2-mercaptoethanol, and 50ug./ml. Gentamicin (Shering Co., Kenilworth, N.J.,U.S.A.).

Diener-Marbrook cultures (Diener and Armstrong, 1969) were set up with cells in a volume of 1 ml. in the inner chamber, and 50ml. of MEM in the flask. Microtitre wells were cultured with varying cell concentrations in 0.2ml. of medium. Costar dishes contained a minimum of 2ml. and a maximum of 10ml. per well.

5) Cell Separation Procedures.

i) Preparation and use of anti-Thy-1 antiserum.

AKR anti-CBA anti-Thy-1 antibody was prepared by a method developed by Dr. I. Ramshaw (personal communication). AKR mice were injected iv with 5 x 10^7 CBA thymocytes, and the serum collected after 7 days was specific for CBA T cells, having no detectable activity against AKR T cells. Anti-Thy-1 treatment of cells consisted of incubating 2 x 10^7 cells/ml. in a 1:5 dilution of anti-Thy-1 antibody on ice for 20 minutes, washing and resuspending the cells in 1ml. of a 1:5 dilution of rabbit complement (supplied by Dr. Pilarski). After 45 minutes in a 37°C. waterbath, the cells were washed and the viable cell counts were estimated by trypan blue exclusion. A more efficient commercial monoclonal anti-Thy-1.2 IgM antibody (New England Nuclear, Lachine, Quebec, Canada) was also available. In this case, 10^7 cells were incubated on ice in a 1:10,000 dilution of the monoclonal antibody for

60 minutes, and agarose-absorbed guinea pig complement (Cohen and Schlesinger, 1970) was added to a final dilution of 1:16. The remainder of the procedure was identical to that described above for the conventional antibody preparation. Cells were also incubated under identical conditions, except that the specific antibody was not added, to ensure that any loss of activity of the treated cells required the presence of the anti-Thy-1 antibody.

ii) T cell enrichment.

Nylon wool (Fenwal Laboratories, Deerfield, Illinois, U.S.A.) was autoclaved a minimum of 3 times in fresh normal saline and lightly packed into a 10cc syringe up to the 7ml. mark. Several volumes of warm Leibovitz medium supplemented with 10% FCS were rinsed through the syringe and the columns were allowed to warm to 37°C. for one hour. A warm cell suspension containing not more than 2×10^8 cells/2ml. was added to the column. The flow was stopped as the last of the cell suspension entered the column by plugging each end of the syringe. These columns were incubated at 37°C. for one hour, then the effluent (T) cells were collected by rinsing the column with 25ml. of Leibovitz medium plus 10%FCS. These were washed and counted by trypan blue exclusion.

iii) Isopaque-ficoll gradients.

Viable cells were separated from dead cells and antigen (RBC) after culture by centrifugation on Isopaque-ficoll gradients (Davidson and Parish, 1975). Four ml. of a 13:5 mixture of 14% ficoll (Ficoll 400, Pharmacia Fine Chemicals, Uppsala, Sweden) in water and 30% sodium metrizoate (Nyegaard and Company, A/S, Oslo, Norway) were placed in a 15ml. polycarbonate tube. Five ml. of cultured cell suspension containing not more than 10^8 viable WBC and not more than 10^{10} RBC was carefully layered over the ficoll solution and these gradients were centrifuged at 2800rpm in an MSE bench-top centrifuge for 20 minutes at room temperature. The viable WBC are collected from the interface of the ficoll solution and the medium, while the dead cells and erythrocytes are spun to the bottom of the tube. Viable cells were washed three times in Leibovitz medium and counted by trypan blue exclusion.

6) Assay of the DTH response.

Viable cells separated on isopaque-ficoll gradients were injected into the left hind footpad of a normal mouse at a dose of 10^7 cells per mouse in a volume of 25ul.. Some mice also received antigen in the 25ul. injection.

Twenty-four hours later, the swelling caused by the sensitised cells was determined as the difference in thickness between the left and the right hind footpads. The footpads were measured with a dial micrometer (Oditest, 649, Schluchtern 1, Germany, F.R.). An anatomical marker on the footpad was used in the positioning of the measuring plates of the micrometer to ensure the repeatability of the measurements, and the plates were allowed to close until no light could be seen between the footpad and the front edge of the measuring plate. Each footpad was measured a minimum of two times, and the mean swelling of mice given 10^7 sensitised cells plus and minus antigen was recorded. The antigen-specific swelling reported is the difference of these means, and, in this case, the standard error is calculated as the square root of the sum of the squares of the standard errors of the swelling obtained with and without antigen.

7) Assay of the humoral response.

The humoral response to a red cell antigen was measured by enumerating the number of PFC by the standard plaque assay (Cunningham and Szenberg, 1968). Fifty ul. of 10% target RBC were combined with 50ul. of a non-toxic dilution (1:5) of guinea pig complement (Flow Laboratories, McLean, Va, USA) in a microwell containing 100ul. of Leibovitz medium (in the case of direct PFC). Twenty ul. of cell suspension were added, the cells were mixed and transferred to Cunningham slide chambers. The slide chambers were sealed with hot pararfin wax, and incubated at 37°C. for a minimum of 60 minutes. Indirect PFC were measured in a similar manner except that, instead of 100ul. of medium, the appropriate wells contained 75ul. of medium and 25ul. of "enhancing serum" (rabbit anti-mouse immunoglobulin). Typically, 1/25th of a culture and 1/200th of a spleen would be assayed.

FGG-specific PFC were assayed by the method of Miller and Warner (1971). FGG-anti-SRBC was coupled, at a dilution 2-fold lower than that required for hemagglutination, by incubating SRBC and this antiserum at 37°C. for 30-60 minutes. PFC responses were then measured against SRBC and FGGSRBC. Fowl gamma globulin can not fix mammalian complement (Cushing, 1965), so the difference in response against these two targets is an estimate of the FGG-specific PFC response.

RESULTS

Introductory Remarks.

The results portion of this thesis has been divided into two main sections. The first consists of preliminary experiments designed to define a culture system in which antigen-specific help for the induction of DTH may be detected and studied. The main ideas tested have been outlined in order to give the reader an idea of how the experiments progressed in the direction they did. In many cases, interesting observations were made, and possible interpretations made as a basis to further develop the system; however, the interpretations were not further substantiated as the purpose of this preliminary work was to establish the system. Major problems have been noted and overcome as the system developed. As many of these were very preliminary experiments, and the low yield of cultured cells was one of the major problems, the results in this section (A) are often obtained from experiments in which very few mice were injected for footpad measurements. The sensitised cells injected without specifically added antigen often caused footpad swelling in the early experiments and this "background" swelling has been recorded for any experiments where it measured more than 20 units.

The second section of these results (B) contain the formal experiments in which the induction of DTH against a xenogeneic erythrocyte in vitro was shown to require an antigen-specific, radioresistant, helper T cell function. The mechanism of action of this helper cell was also studied and shown to act by the linked recognition of antigenic determinants. In these experiments, 10 mice have been injected per experimental group in each experiment, 5 with 10^7 sensitised cells and antigen and 5 with 10^7 sensitised cells alone.

A1) Conditions for the induction of DTH *in vitro*

i) Two chosen antigens do not cross-react

In this study, culture conditions have been investigated to establish the requirements for the induction of DTH *in vitro*, and how they are related to, or differ from, the requirements for the induction of humoral immunity. It was necessary to have two non-cross-reacting antigens that could be physically linked to one another, or not, at will, in order to perform the planned linked recognition experiments. BRBC and FGG were chosen because there is no evidence of background immune response to these antigens, and hence responses against them are not observed on culturing cells in the absence of antigen. The cross-reactions between the humoral responses to these two antigens were examined to determine whether they were grossly cross-reactive. Mice were injected iv with either 4×10^8 BRBC, or 4×10^8 FGGBRBC conjugates, the FGG having been coupled to the erythrocytes with chromic chloride. Spleen cells from these mice were assayed for PFC against SRBC, FGGSRBC, and BRBC monolayers 4 days later. The results are shown in Table I, and while both groups of mice respond to BRBC, the only response to FGGSRBC over the sheep background response occurs in those mice injected with the FGGBRBC conjugate. This is preliminary evidence that the FGG and the BRBC do not grossly cross-react, at least at the antibody level in the whole animal, since immunisation with burro erythrocytes did not increase the FGG PFC response. It is also evident from these results that the FGG coupled to the BRBC does not completely obscure the burro-specific determinants since the PFC response against burro is comparable to the response against FGG.

Experiment 3 of Table I also included Diener-Marbrook 4-day cultures with 1.5 x 10^7 normal spleen cells plus 10^7 BRBC, resulting in a PFC resonse against BRBC. The same type of cultures incubated with FGGBRBC did not produce any significant FGG- or BRBC-specific PFC response (Table I). Other cultures in the same experiment contained 1.5 x 10^7 normal spleen cells plus 10^9 BRBC and resulted in the induction of DTH against BRBC after 6 days in culture; and when FGGBRBC conjugates were used as antigen, some DTH was induced against BRBC and FGG (Table II).

TABLE I

Day 4 PFC response *in vivo* or *in vitro*

Antigen	PFC^1		
	SRBC	FGGSRBC	BRBC
Exp1			
BRBC^2	800(566)	200 (141)	8800(2600)
FGGBRBC^2	200(141)	16600(5800)	17400(4667)
Exp2			
BRBC^2	0 (0)	200 (115)	23600(6582)
FGGBRBC^2	200(115)	9200(1500)	11200(3695)
Exp3			
BRBC^2	200(115)	200 (115)	21400(2200)
FGGBRBC^2	200(115)	12800(3350)	17000(1620)
BRBC^3	550(210)	250 (80)	5800(1765)
FGGBRBC^3	1035(220)	900 (280)	550 (180)
----^{3}	500 (71)	775 (175)	0 (0)

1)Numbers represent mean number of PFC (plaque-forming cells) per spleen, or per culture, and the numbers in parentheses represent the standard errors. Experiment 1 - 3 mice per group; Experiments 2,3 - 4 mice or cultures per group.

2)*In vivo* experiments. Mice were injected iv with 4 x 10^8 BRBC or FGGBRBC conjugates 4 days previously, and assayed for BRBC- and FGG-specific PFC.

3)*In vitro* experiments. Cultures were assayed at 4 days for BRBC- or FGG-specific PFC responses after culture of 1.5 x 10^7 normal spleen cells and 10^7BRBC, 10^7 FGGBRBC conjugates, or no antigen.

TABLE II

DTH response to FGG or BRBC after 6 days in culture

Antigen (in culture)	Swelling[1]		
	BRBC	FGGMRBC	no antigen
A) BRBC	78 (4)	N.D.[2]	26(13)
B) FGGBRBC	52(13)	41(15)	7 (1)
C) no antigen	40 (8)	22(10)	N.D.

1)Mean swelling of 3 mice injected with 10^7 sensitised cells and 5 x 10^7 BRBC, 5 x 10^7FGGMRBC, or no antigen. Swelling is measured in units of 10^{-2}mm., and the numbers in parentheses represent the standard errors.

2)Not done.

ii) Help for thymocytes.

In order to demonstrate a requirement for help, one must set up an experimental system where one suspects help is limiting, and add a source of antigen-primed cells that will contribute to the antigen-specific induction of immune reactivity, but not contribute functional precursor cells to the cultures. Irradiated spleen cells from antigen-primed animals have been used in the past to provide such a source of helper avtivity for the humoral response (Kettman and Dutton, 1971) and for the cytotoxic T cell response (Pilarski, 1977). Irradiation does not allow cells to divide and differentiate, but cells that are fully differentiated at the time of irradiation can apparently function until their death. It was decided to use thymocytes as a source of DTH precursor cells and to add irradiated spleen cells from mice primed 4 days previously with 5 x 10^5 BRBC iv as a source of helper cells. Table III shows the results of a preliminary attempt to induce DTH in this system. Although the swelling activity induced is small, the results indicate that thymocytes cultured with irradiated spleen cells from specifically primed mice induce some swelling activity if cultured with antigen while those thymocytes cultured with normal irradiated spleen cells do not. The thymocytes are necessary in these cultures as shown in line B, and any surviving irradiated primed spleen cells are not likely to be responsible for the swelling observed.

Table IV shows the results of a limited titration of irradiated spleen cells from mice primed with 10^6 BRBC iv 6 days before their spleens were removed and used to help induce DTH reactivity with thymocytes. The antigen-specific swelling can be seen to be increased only in those cultures containing 10^7 irradiated primed spleen cells. Lower numbers of these primed helpers cannot be supplemented to 10^7 with irradiated normal cells to provide effective help.

TABLE III

DTH response of thymocyte cultures containing irradiated spleen cells from normal or BRBC-primed mice.

	thymocytes	irradiated cells[1]		BRBC	swelling[3]	
		normal	primed[2]		+BRBC	-ag
A)	10^7	---	10^7	10^9	29(6)	2(3)
B)	---	---	10^7	10^9	-4(5)	N.D.[4]
C)	10^7	10^7	---	10^9	10(9)	10(1)
D)	10^7	---	10^7	---	6(5)	3(4)

1) Irradiated cells received 1500 rad. of gamma irradiation.
2) Mice were primed iv with 5 x 10^5 BRBC 4 days previously.
3) Mean swelling in 10^{-2}mm. units of 2-3 mice injected with 10^7 sensitised cells and 5 x 10^7 BRBC or 10^7 sensitised cells alone (no antigen).
4) Not done. In this experimental group, only 3 x 10^5 viable cells were harvested after 6 days of culture from an input of 1.8 x 10^8. These were injected equally into 2 mice to ensure these were not potent DTH effector cells that had survived after the irradiation treatment.

TABLE IV

Titration of irradiated helper cells from primed mice

	thymocytes	irradiated cells[1]		BRBC	antigen-specific swelling[3]
		normal	primed[2]		
A)	10^7	95×10^5	5×10^5	10^9	-8(10)
B)	10^7	8×10^6	2×10^6	10^9	15(15)
C)	10^7	---	10^7	10^9	63 (8)

1) Irradiated cells received 1500 rad. of gamma irradiation.

2) Mice were primed iv with 10^6 BRBC 6 days previously.

3) BRBC-specific swelling is calculated as the mean swelling (in 10^{-2}mm. units) of 3 mice given 10^7 sensitised cells and 5×10^7 BRBC minus the mean swelling of 3 mice given 10^7 sensitised cells without antigen. The standard error of the difference is represented by the numbers in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.

iii) Can priming to one antigen increase the DTH response to a linked determinant?

Results of other preliminary experiments to see if priming to one antigen might help the induction of DTH to an antigen physically linked to it are shown in Tables V, VI and VII. The results in Table V are from an experiment in which mice were primed with 4 x 10^5 BRBC iv 2 days before their spleens were removed and cultured (without irradiation) with either no antigen or FGGBRBC. After 6 days in culture the DTH was measured against both BRBC and FGG. The FGG-specific DTH was elicited both with alum-precipitated FGG and with FGGMRBC, and these gave equivalent swelling when injected with cells cultured with FGGBRBC. The burro-primed cells cultured without antigen did not give significant swelling when injected with either form of the FGG antigen, and the burro response indicates that even primed cells require antigen in culture in order to develop maximum antigen-specific swelling in the footpad after 6 days incubation.

The results in Tables VI and VII are from experiments in which mice were twice primed with 0.5 mg. FGG iv 3 and 6 days before their spleens were irradiated and used as a source of FGG-specific helper cells for the induction of burro-specific DTH in thymocytes cultured with FGGBRBC conjugates in Diener-Marbrook flasks (Table VI) or Microtitre trays (Table VII). Although the results were somewhat encouraging, the yield of viable cells from thymocytes after 6 days of culture is very low, and the swellings obtained were not large enough to be reliably estimated when less than 10^7 cultured cells were injected into each footpad. The number of cells required after 6 days of culture is of the order of 10^8 (10^7 per mouse, 5 mice + or - antigen). The flasks require 50 ml. of MEM each, and the yield of thymocytes after 6 days of culture is typically around 10%. This means that meaningful experiments using this culture system would be very cumbersome and very expensive. Thymocytes survive even less well in Microtitre trays. Attempts to use lymph node cells as responder cells, or to use nylon wool purified irradiated primed spleen cells as a helper source, or to treat these with anti-immunoglobulin and complement in order to obtain a more efficient antigen-specific helper population (without interference from antibody), did not result in increased antigen-specific swelling reactivity being induced in culture. Spleen cells survive well in culture and it was thought these might be used at limiting density as a source of precursor DTH cells.

TABLE V

BRBC-primed spleen cells increase the DTH response to FGG after incubation with FGGBRBC conjugates.

Primed spleen[1]	FGGBRBC[2]	swelling[3]			
		+BRBC	+FGGMRBC	+FGGap	-ag
A)1.5 x 10^7	---	44(4)	21(1)	19(0)	N.D.[4]
B)1.5 x 107	109	118(8)	51(4)	55(8)	13(2)

1) Mice were primed iv with 4 x 10^5 BRBC 2 days previously.

2) FGG was coupled to BRBC by the chromic chloride technique.

3) Swelling is the mean of 2-4 mice given 10^7 sensitised cells and 5 x 10^7 BRBC, 10^7 FGGMRBC, 5ul. of a 1:8 dilution of alum precipitated FGG in saline, or no antigen. Numbers in parentheses represent standard errors.

4) Not done.

TABLE VI

FGG-primed spleen cells increase the DTH response to BRBC after culture with FGGBRBC conjugates in Diener-Marbrook flasks.

	thymocytes	irradiated cells[1]		FGGBRBC[3]	antigen-specific swelling[4]	
		normal	primed[2]		day 5	day 6
Exp1						
A)	10^7	7×10^6	3×10^6	10^7	44 (6)	97(18)
B)	10^7	---	10^7	10^7	35 (8)	42(10)
Exp2						
C)	10^7	10^7	---	10^7	28 (1)	45(44)
D)	10^7	9×10^6	10^6	10^7	63 (5)	54(15)
E)	10^7	7×10^6	3×10^6	10^7	23 (3)	35(21)
F)	10^7	---	10^7	10^7	8 (6)	7 (1)

1) Irradiated cells received 1500 rad. of gamma irradiation.

2) Mice were primed iv with 0.5mg. of FGG 3 and 6 days previously.

3) FGG was coupled to BRBC by the chromic chloride technique.

4) BRBC-specific swelling is calculated as the mean swelling (in 10^{-2}mm. units) of 2-4 mice given 10^7 sensitised cells and 5×10^7 BRBC minus the mean swelling of 2-4 mice given 10^7 sensitised cells without antigen. The standard error of the difference is represented by the numbers in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.

TABLE VII

FGG-primed spleen cells increase the DTH response to BRBC after culture with FGGBRBC conjugates in Microtitre trays.

	thymocytes	irradiated cells[1] normal	irradiated cells[1] primed[2]	FGGBRBC[3]	antigen-specific swelling[4] day 5	antigen-specific swelling[4] day 6
A)	10^6	$2.5x10^5$	---	10^5	0 (1)	25 (1)
B)	10^6	$2.4x10^5$	10^4	10^5	33 (2)	21(12)
C)	10^6	$2.2x10^5$	$3.0x10^4$	10^5	41 (8)	47 (1)
D)	10^6	$1.5x10^5$	10^5	10^5	37(27)	20 (4)
E)	10^6	---	$2.5x10^5$	10^5	14 (6)	8 (2)

1) Irradiated cells received 1500 rad. of gamma irradiation.

2) Mice were primed iv with 0.5mg. of FGG 3 and 6 days previously.

3) FGG was coupled to BRBC by the chromic chloride technique.

4) BRBC-specific swelling is calculated as the mean swelling (in 10^{-2}mm. units) of 1-2 mice given 10^7 sensitised cells and 5 x 10^7 BRBC minus the mean swelling of 1-2 mice given 10^7 sensitised cells without antigen. The standard error of the difference is represented by the numbers in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.

iv) The relationship between spleen cell numbers, and the induction of humoral and DTH responses.

Varying densities of normal spleen cells were cultured in Diener-Marbrook flasks with a single antigen concentration to determine the dependence of the induction of humoral and DTH responses on cell density. It can be seen from Table VIII that, under these culture conditions, the PFC response peaks early (day 4) and is only present in the most dense cultures, while the DTH reactivity appears later and requires less dense culture conditions. (Antigen-specific swelling was not measured on day 4 in this experiment but has never been detected in this laboratory after 4 days of culture with unprimed responder cells.) These cultures show a tendency for exclusiveness of the class of immune response induced under particular conditions, and PFC are induced only in relatively dense normal spleen cell cultures.

TABLE VIII

Relationship between cell density and the induction of DTH or PFC responses in culture.

Spleen cells	BRBC	PFC[1]			swelling[2]	
		day4	day5	day6[3]	day5	day6
A)1.5×10^7	10^7	8350(637)	850(100)	1450	35(5)	32(12)
B)7.5×10^6	10^7	1150(180)	100 (50)	250	23(7)	35 (1)
C)3.25×10^6	10^7	600 (41)	50 (29)	100	20(6)	5 (9)

1) Numbers represent mean number of PFC (plaque-forming cells) per 3-7 Diener-Marbrook flasks per day, and the numbers in parentheses represent the standard errors.
2) BRBC-specific swelling is calculated as the mean swelling (in 10^{-2}mm. units) of 2-4 mice given 10^7 sensitised cells and 5×10^7 BRBC minus the mean swelling of 2-4 mice given 10^7 sensitised cells without antigen. The standard error of the difference is represented by the numbers in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.
3) Day 6 PFC response was estimated from a pool of cultures, so no errors are available.

A similar pattern is seen when lethally irradiated mice are used as tissue culture vessels, different groups being injected with increasing numbers of normal spleen cells and a constant dose of FGG as antigen (Table IX). This is analagous to experiments performed by Dr. Bretscher using BRBC as antigen (Bretscher, manuscript submitted). The mice were irradiated with 850 rad. of gamma irradiation, injected with 50 units of heparin ip, and variable doses of normal spleen cells were injected iv along with 0.5 mg. of FGG. Three days later, the mice were boosted again with 0.5 mg. FGG iv. After 6 days the spleens of these mice were removed and the cells tested for DTH reactivity in the footpads of normal mice. The PFC response was also tested. The lowest spleen cell number transferred is induced to DTH reactivity while larger numbers of spleen cells transferred are induced to express less DTH reactivity (Table IX). There was no significant FGG-specific PFC response induced in any of these animals 6 days after the adoptive transfer. Analogous experiments in which larger numbers of normal spleen cells were transferred did result in a PFC response with no detectable DTH activity (results not shown).

TABLE IX

Relationship between cell numbers transferred to lethally irradiated mice and the induction of DTH

	normal spleen[1]	FGG-specific swelling[2]
A)	10^7	64 (9)
B)	2×10^7	53(18)
C)	3×10^7	56(12)
D)	4×10^7	17 (5)

1) Recipient mice were irradiated with 850 rad. of gamma irradiation, injected with 50 units of heparin ip, then the stated number of normal spleen cells was injected iv. These mice were injected on the day of transfer and again 3 days later with o.5mg. of FGG iv. Assays were performed 6 days after the adoptive transfer.

2) Spleen cells harvested from the adoptive transfer recipients were injected at a dose of 10^7 viable cells with or without 5ul. of a 1:8 dilution of alum precipitated FGG in saline. Numbers in parentheses represent the standard errors calculated as the square root of the sum of the squares of the standard errors with and without antigen.

v) Comparison of culture systems for DTH induction.

Spleen cells were routinely used as responding cells for subsequent experiments, and these were tested at varying densities in Diener-Marbrook flasks, Costar dishes (35mm) and Microtitre trays (6mm) for comparison. Table X shows the results of measuring the swelling activity which can be induced in each of these systems, and it was noted that the swelling obtained in the footpads of mice injected with sensitised cells without deliberately added antigen varied considerably. The Costar dishes offered many advantages in that they were easy to handle and harvest, the yields were reasonable, and the MEM required was only 10 ml. per culture. Various spleen cell densities were studied in the Costar system to find a limiting dose of spleen cells that might be used for helper cell detection in the induction of DTH. Table XI shows the swelling induced after varying cell densities are cultured for 6 days with 10^9 BRBC. The production of swelling activity requires a minimum of 10^7 normal spleen cells with this antigen concentration, peaks at approximately 1.5×10^7 cells per Costar well, and requires antigen to be present in culture and in the footpad. In order to reduce the non-specific swelling noted previously, the injection syringe was washed carefully with alcohol after each use and stored full of alcohol; also, the needle was either changed or sharpened before injections. Those cells cultured with very high antigen concentrations still caused some swelling when injected without specifically added antigen, but this is probably largely due to antigen carry over. The ficoll gradients were unable to efficiently remove all traces of this highly concentrated antigen without severely depleting the numbers of viable cells.

TABLE X

Comparison of culture systems for the induction of DTH

system	spleen cells	BRBC	swelling[1]	
			+BRBC	-ag
Diener-Marbrook flasks	1.5×10^7	10^9	94 (9)	39[2]
	1.5×10^7	---	40(13)	27[2]
Microtitre trays	10^6	10^7	91 (6)	25(14)
	10^6	---	42(18)	44[2]
	5.0×10^5	10^7	154(13)	49 (9)
	5.0×10^5	---	41 (3)	18 (5)
	2.5×10^5	10^7	116(11)	63(13)
	2.5×10^5	---	33(11)	24 (8)
Costar dishes	10^7	10^9	103(22)	64(16)
	10^7	---	15 (1)	19[2]
	4.0×10^6	10^9	81(20)	50[2]

1) Swelling is the mean of 1-4 mice given 10^7 sensitised cells and 5×10^7 BRBC or 10^7 sensitised cells alone (no antigen). Numbers in parentheses represent standard errors.

2) Only 1 mouse per group. No standard error available.

TABLE XI

Induction of DTH in normal spleen cells by high concentrations of antigen in culture

Spleen cells	BRBC	Swelling[1]	
		+BRBC	-ag
A) 3×10^7	10^9	66(6)	39 (9)
B) 2×10^7	10^9	55(5)	40 (2)
C) 1.5×10^7	10^9	92(4)	58 (2)
D) 10^7	10^9	83(9)	51(14)
E) 5×10^6	10^9	46(6)	31 (0)
F) 1.5×10^7	---	39(3)	31 (1)
G) 5×10^6	---	31(1)	N.D.[2]

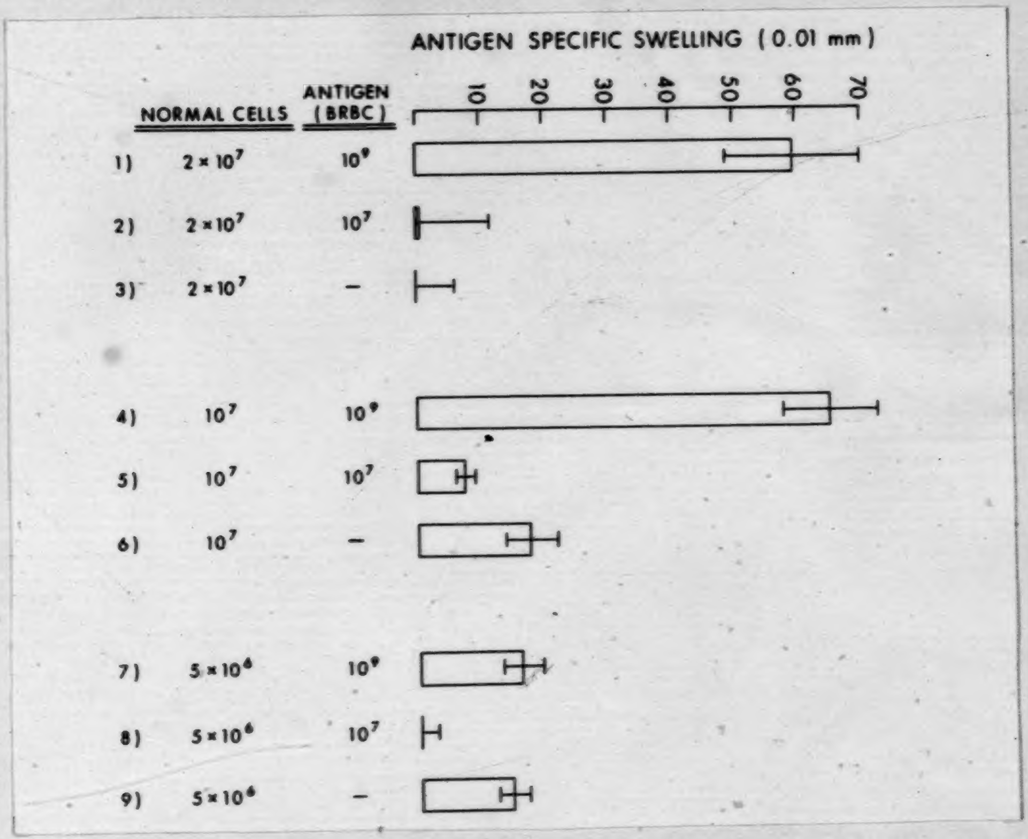
1) Mean swelling in 10^{-2} mm. units of 2-4 mice injected with 10^7 sensitised cells and 5×10^7 BRBC or 10^7 sensitised cells alone (no antigen). Numbers in parentheses represent the standard errors.

2) Not done.

vi) Dependence of *in vitro* induction of DTH on cell density and antigen concentration

Figure 1 shows the results of a more complete study of varying cell densities and antigen concentrations on the antigen-specific swelling induced in spleen cells cultured for 6 days in Costar dishes. A high antigen concentration (10^9RBC per well) allows the induction of DTH in cultures containing a minimum of 10^7 normal spleen cells, but not in cultures containing one-half that number. DTH is effectively induced at doses of 1-2 x 10^7 normal spleen cells per well if 10^9 BRBC are provided as antigen, but not with one one-hundredth of that concentration. In fact, at the lower antigen concentration with high normal spleen cell densities, PFC are induced instead. The PFC response of these cultures was assayed on days 4 and 5, and was really only evident in those cultures containing 2 x 10^7 responder cells and 10^7 BRBC (Table XII). 10^7 BRBC provide sufficient antigen to induce DTH in Costar wells that contain 3-3.5 x 10^7 normal spleen cells per well (data not shown), but these dense cultures require a change of medium during the incubation to ensure a reasonable yield of cells after 6 days. These observations are understandable if cellular collaboration between DTH precursor cells and helper T cells is essential for the induction of DTH, and the cooperative event becomes more infrequent as the density of spleen cells decreases, especially at the lower antigen concentrations.

Figure 1



Dependence of in vitro induction of DTH on cell density and antigen concentration.

TABLE XII

Induction of PFC response in culture with varying cell densities and antigen concentrations.

	Spleen cells	BRBC	PFC[1]	
			day 4	day 5
A)	2×10^7	10^9	20 (13)	163 (69)
B)	2×10^7	10^7	800(125)	1425(222)[2]
C)	2×10^7	---	50(125)	44 (41)
D)	10^7	10^9	0 (0)	0 (0)
E)	10^7	10^7	150 (41)	144(101)
F)	10^7	---	145 (93)	150(101)
G)	5×10^6	10^9	0 (0)	0 (0)
H)	5×10^6	10^7	0 (0)	0 (0)
I)	5×10^6	---	0 (0)	0 (0)

1) Numbers represent the mean of PFC (plaque-forming cells) per culture, (5-6 samples), and the numbers in parentheses represent the standard errors. This table and Figure 1 are from the same experiment.

2) PFC response for this group on day 6 was 800(120).

vii) Summary

The results of these preliminary experiments indicate that the Costar culture system using spleen cells as responder cells is the best system of those investigated. DTH can be induced in these cultures under certain conditions, and these conditions of normal spleen cell density and antigen concentration can be adjusted to provide cultures where cellular cooperation may very well be limiting. To test whether specific cooperation is indeed limiting in these cultures, one can add a source of putative antigen-specific helper cells and test whether these allow the induction of DTH. These putative helper cells could be obtained from spleen cells from antigen-primed mice. Preliminary experiments, both those reported here and numerous others, indicate that these will provide helper activity when irradiated, while equivalent numbers of irradiated normal spleen cells will not.

Other preliminary results in this section indicate that the antigens chosen (protein and erythrocyte) are probably not cross-reactive, and that DTH specific for either antigen can be elicited. Mice primed to one of these antigens tend to provide help for the induction of DTH specific for the other antigen in the presence of the conjugate, but these experiments only indicate, not demonstrate, specificity of the helper cell(s).

A2) Help for low spleen cell density and high antigen concentration.

i) The system.

The addition of irradiated antigen-specific helper cells to those cultures containing either 5 x 10^6 normal spleen cells and 109 BRBC or 107 normal spleen cells and 10^7 BRBC should allow the induction of DTH in these cultures, if the collaborative event is limiting. The lower dose of spleen cells was chosen for study first. The DTH and PFC responses after 4,5 and 6 days of culture are shown in Table XIII. Clearly there is no significant DTH or PFC response on any day at any dose under these conditions.

TABLE XIII

Induction of DTH and PFC responses in cultures containing 5 x 10^6 normal spleen cells and varying antigen concentrations.

antigen	antigen-specific swelling[1]			PFC[2]		
	day4	day5	day6	day4	day5	day6
A) 10^9	13(3)	11(2)	17(3)	0 (0)	15 (11)	45(26)
B) 10^7	14(4)	15(5)	0(2)	490(127)	595(105)	190(59)
C) ---	13(2)	6(5)	15(2)	110 (37)	75 (44)	44(11)

1) Antigen(BRBC)-specific swelling is calculated as the mean swelling (in 10^{-2} mm. units) of 3-5 mice given 10^7 sensitised cells plus 5 x 10^7 BRBC minus the mean swelling of 3-5 mice given 10^7 cells without antigen. The standard error of the differences is represented by the number in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.

2) Numbers represent mean of PFC (plaque-forming cells) per 5 cultures per group. The numbers in parentheses represent the standard errors.

ii) Conditions for obtaining primed cells that act as helpers in the induction of DTH.

Irradiated spleen cells from mice primed 4 days previously with 10^9 or 2×10^5 BRBC iv were used to try to help induce a DTH response in cultures containing 5×10^6 normal spleen cells and 10^9 BRBC. The results are shown in Table XIV. The helper cells taken from mice primed with high doses of BRBC do not help induce as high levels of DTH reactivity as those taken from mice primed with lower doses. It should be noted that, of many attempts to induce DTH *in vitro* using irradiated spleen cells from mice primed to mount a humoral response, this is the only experiment in which appreciable levels of help could be demonstrated from such cells. Also, the number of helper cells from mice primed with the higher doses of antigen is extremely critical since over an 8-fold increase, these cells first help and then suppress DTH induction in these cultures. There were no PFC at all on day 6 in the cultures with help from the mice primed with high doses of BRBC.

Spleen cells from mice used in the adoptive transfer induction of FGG-specific DTH did not function as specific irradiated helper cells for the *in vitro* induction of BRBC-specific DTH when cultured with FGGBRBC conjugates.

It was investigated whetner the addition of large numbers of irradiated cells might interfere with the induction of antigen-specific swelling activity in culture. Spleen cells from mice primed 2 days previously with low doses of BRBC iv (2×10^5) were cultured with increasing numbers of irradiated normal spleen cells, up to 4×10^7 per culture, and there was no inhibition of the response at any dose tested.

TABLE XIV

Helper cells for low spleen cell densities (5×10^6) and high antigen concentrations (10^9).

	irradiated cells[1]			BRBC	swelling[2]	
	normal	high	low		+BRBC	-ag
A)	----	4×10^4	----	10^9	19 (5)	7(4)
B)	----	8×10^4	----	10^9	49 (2)	17(1)
C)	----	1.6×10^5	----	10^9	59(14)	16(5)
D)	----	3.2×10^5	----	10^9	20 (6)	12(1)
E)	----	6.4×10^5	----	10^9	17 (2)	18(2)
F)	1.6×10^5	----	----	10^9	19 (3)	16(4)
G)	----	----	1.6×10^5	---	10 (2)	10(2)
H)	----	----	3×10^5	10^9	44 (4)	36(3)
I)	----	----	10^6	10^9	76 (7)	31(4)
J)	----	----	3×10^6	10^9	78 (5)	29(3)
K)	----	----	10^7	10^9	87 (2)	35(5)
L)	10^6	----	----	10^9	41 (3)	29(5)
M)	10^7	----	----	10^9	36 (3)	34(3)
N)	----	----	3×10^6	---	21 (2)	21(2)

1) Irradiated cells received 1500 rad. of gamma irradiation before being added to the cultures in the numbers indicated. "High" irradiated cells came from mice primed with a high dose of BRBC (10^9) iv 4 days previously; "low" irradiated cells came from mice primed with a low dose of BRBC (2×10^5) iv 2 days previously.

2) Mean swelling in 10^{-2}mm. units of 3-5 mice injected with 10^7 sensitised cells and 5×10^7 BRBC or 10^7 sensitised cells alone (no antigen). The numbers in parentheses represent the standard errors.

iii) Kinetics of priming.

Studies on the kinetics of the appearance of helper cells after *in vivo* priming show that effective help for DTH is present even on day 2 after mice were primed with 2 x 10^5 BRBC iv (Table XV). The help obtained is not increased 3 or 4 days after iv priming with BRBC. Those cultures containing normal irradiated spleen cells do not result in any significant antigen-specific swelling, and those containing the most effective irradiated helper cells do not result in any antigen-specific swelling unless antigen is provided during the culture period. Although the background swellings are very high in the cells harvested from these cultures with high antigen concentrations, this effect can be shown to be largely due to incomplete antigen removal by the isopaque-ficoll gradients (rows B vs J, Table XV). The viable cell loss during either repeated isopaque-ficoll gradients or low dose loading of these gradients made it impractical to use these techniques routinely for complete antigen removal. Using lower antigen doses in culture with 10^7 or 10^6 irradiated primed spleen as a source of helper cells did not allow 5 x 10^6 normal spleen cells to be induced.

TABLE XV

Kinetics of the appearance of *in vivo* helpers for low spleen cell densities (5×10^6) following iv priming with 2×10^5 BRBC.

	irradiated cells[1]				BRBC	swelling[2]	
	normal	day2	day3	day4		+BRBC	-ag
A)	---	10^6	---	---	10^9	86 (5)	42 (1)
B)	---	10^7	---	---	10^9	94 (6)	68 (3)[4]
C)	---	---	10^6	---	10^9	54 (4)	47 (6)
D)	---	---	10^7	---	10^9	82 (4)	69(13)
E)	---	---	---	10^6	10^9	87 (3)	57 (3)
F)	---	---	---	10^7	10^9	93 (4)	61 (4)
G)	---	---	---	---	10^9	37 (2)	20 (4)
H)	10^7	---	---	---	10^9	41(11)	25 (5)
I)	---	10^7	---	---	---	13 (3)	2[3]
J)	---	10^7	---	---	10^9	92 (3)	33(11)[4]

1) Irradiated cells received 1500 rad. of gamma irradiation before being added to the cultures in the numbers indicated. These came from unprimed mice or mice primed with 2×10^5 BRBC iv 2,3 or 4 days previously.

2) Mean swelling in 10^{-2}mm. units of 1-5 mice injected with 10^7 sensitised cells and 5×10^7 BRBC or 10^7 sensitised cells alone (no antigen). The numbers in parentheses represent the standard errors.

3) One mouse only injected in this group, so no error estimate available.

4) J is the same experimental group as B, except that it was loaded on more ficoll gradients to decrease the antigen carry over in the "negative" footpads. Cultures with these very high antigen concentrations often tend to have RBC retained at the isopaque-ficoll interface.

iv) Summary

In this section, help for low spleen cell density and high antigen concentration cultures has been investigated. It was found that, in this culture system, lymphoid cells could be induced to yield DTH reactivity by the addition of primed irradiated helper cells. Spleen cells from mice primed iv with low doses of antigen gave more consistent helper activity than those given higher doses iv. Major problems with the use of this particular system include an inability to efficiently remove antigen from the spleen cells before footpad injection, and, again, a low yield of spleen cells after culture with these very high antigen concentrations.

A3) Help for higher spleen cell density and lower antigen concentration.

i) Low dose iv priming.

In an attempt to reduce the antigen carry over into the control footpads, and to increase further the yield of viable cells after 6 days of culture, some experiments were perrormed to try to help induce DTH in cultures with a higher density of spleen cells (10^7) and with less antigen (10^7 BRBC). Helper cells were obtained from mice primed 2 days previously with 2 x 10^5 BRBC iv. The results are shown in Table XVI. The cultures containing 2 x 10^7 normal spleen cells can be seen to result in DTH reactivity with the addition of normal irradiated spleen cells. Those cultures containing 10^7 normal spleen cells do not result in DTH reactivity under these conditions, unless primed irradiated cells and antigen are added. It can be seen that, although the antigen-specific swelling is not very large, the swelling in footpads injected without antigen has been reduced to a reasonable size, so that it is no longer a major concern.

2

TABLE XVI

Induction of DTH in cultures containing higher densities of normal spleen cells and lower antigen concentrations.

spleen cells	irradiated cells[1]		BRBC	swelling[2]	
	normal	primed		+BRBC	-ag
A) 10^7	---	10^7	10^7	43 (7)	11(5)
B) 2×10^7	---	10^7	10^7	54 (9)	11(2)
C) 10^7	10^7	---	10^7	18 (3)	11(1)
D) 2×10^7	10^7	---	10^7	49 (4)	9(3)
E) 2×10^7	---	10^7	---	15 (4)	7(3)

1) Irradiated cells received 1500 rad. of gamma irradiation before being added to the cultures in the numbers indicated. These came from unprimed mice or mice primed with 2×10^5 BRBC iv 2 days previously.

2) Mean swelling in 10^{-2}mm. units of 5 mice injected with 10^7 sensitised cells and 5×10^7 BRBC or 10^7 sensitised cells alone (no antigen). The numbers in parentheses represent the standard errors.

ii) Priming with FGG

Normal spleen cell cultures containing the FGGBRBC conjugate, prepared by coupling FGG with chromic chloride, sometimes supported the induction of DTH to BRBC, even in the absence of help. It is known from other studies (Ramshaw and Eidinger, 1977) that spleen cells cultured without antigen show swelling activity to bromelain-treated MRBC, and it was considered possible that the coupled BRBC might be damaged such that they bore determinants that cross-react with bromelain-treated MRBC. A fowl anti-BRBC antiserum was prepared and this was coupled to the BRBC by its antigen-specific receptors and used as a conjugate, since it was felt that the specific antibody would not disturb the erythrocyte membrane as did the chemical coupling procedure. Cells coupled in this way did not support the induction of significant DTH to BRBC unless specific help was provided as described below.

Spleen cells from normal mice or mice primed with 0.5 mg. of FGG iv 2 days previously were cultured (without irradiation) in Costar dishes with BRBC or FGGBRBC conjugates prepared with the fowl anti-burro antiserum. The results (Table XVII) show that burro-specific DTH is induced only in those cultures in which primed cells are present and the conjugate is provided as antigen. It can be seen that equivalent levels of antigen-specific swelling have been induced against both BRBC and FGG. In these experiments there was no FGG present at all in those cultures containing BRBC as antigen, so it was not clear whether the helper T cells acted by the linked recognition of determinants on the molecule.

TABLE XVII

The induction of BRBC- or FGG-specific DTH in spleen cells from normal or FGG-primed mice after incubation with FGGBRBC, BRBC or no antigen.

FGG-primed spleen[1]	normal spleen	antigen in culture[2] (10^7)	swelling[3]		
			BRBC	FGGap	-ag
Exp1					
A) 2 x 10^7	---	FGGBRBC	59 (5)	54(10)	15(5)
B) 10^7	---	FGGBRBC	61(11)	---	11(2)
C) 10^7	---	BRBC	26 (4)	---	6(6)
Exp2					
D) 10^7	---	FGGBRBC	52 (6)	58 (6)	11(4)
E) 10^7	---	BRBC	16 (1)	17 (4)	3(1)
F) 10^7	---	---	15 (2)	21 (3)	4(3)
G) ---	10^7	FGGBRBC	17 (4)	16 (3)	10(2)
H) ---	10^7	BRBC	15 (3)	---	7(1)
I) ---	10^7	---	9 (1)	---	7(1)

1) Mice were primed with 0.5mg. FGG iv 2 days previously. These cells have not been irradiated.

2) FGG was coupled to the BRBC by incubating the erythrocytes with sub-agglutinating amounts of specific fowl-anti-BRBC antiserum for 30-60 minutes at 37°C..

3) Mean swelling of 5 mice injected with 10^7 sensitised cells and 5 x 10^7 BRBC, 5ul. of a 1:8 dilution of alum precipitated FGG, or no antigen. Swelling is measured in units of 10^{-2}mm., and the numbers in parentheses represent the standard errors.

iii) sc priming

It seemed likely that an even better source of help for the induction of DTH might be lymphocytes from an animal expressing optimal DTH reactivity to the antigen. Mice primed to give a stronger, more stable DTH reaction to BRBC might contribute still more efficient helper cells than had been obtained so far. Lagrange, Mackaness and Miller (1974) found more stable and longer lasting DTH reactions in mice injected sc rather than those injected iv with xenogeneic red cells. Mice were primed sc in the footpad with various doses of BRBC, and the optimum dose for DTH induction in the whole animal was found to be 5 x 10^8, in agreement with the general observations of Lagrange et al.. Spleen cells from mice so primed with 5 x 10^8 BRBC 2, 4 or 6 days previously were tested for helper activity in culture. Two doses of antigen were tested with these irradiated helper cells, as shown in Table XVIII. These results are clearly better than those previously obtained using irradiated primed cells from iv injected mice as a source of help. The background swelling now is almost never more than 20 units, except occasionally when the DTH is very strong (e.g., row C had a background of 34 units, but this is unusually high). Additional titrations were performed to ensure the optimum dose of helper cells was

10^7, and a final titration showed that the cultures induce maximal activity when the volume per culture is reduced to 4 ml.. Similar observations were made on the optimal dose of helper cells, the time of appearance after *in vivo* priming, and the relative efficiency of different routes of priming on the production of helper cells for the antigen CRBC. FGG, however, did not induce efficient helper activity when injected sc either as the FGGMRBC conjugate or as alum precipitated FGG. Injection of 0.5mg. of FGG iv did produce efficient FGG-specific helper cells in 2-4 days, and the optimum dose was still 10^7 cells per culture.

TABLE XVIII

Kinetics of the appearance of *in vivo* helpers for the induction of DTH following sc priming with 5×10^8 BRBC.

	normal spleen	irradiated cells[1]			BRBC	swelling[2]
		day2	day4	day6		
A)	10^7	---	---	---	10^7	1 (5)
B)	10^7	10^7	---	---	10^7	8 (6)
C)	10^7	---	10^7	---	10^7	91(32)
D)	10^7	---	---	10^7	10^7	99(11)
E)	10^7	---	---	---	10^8	4 (5)
F)	10^7	10^7	---	---	10^8	-1 (7)
G)	10^7	---	10^7	---	10^8	61(16)
H)	10^7	---	---	10^7	10^8	122 (9)

1) Irradiated cells received 1500 rad. of gamma irradiation before being added to the cultures in the numbers indicated. These came from mice primed with 5×10^8 BRBC sc 2,4 or 6 days previously.

2) BRBC-specific swelling is calculated as the mean swelling (in 10^{-2} mm. units) of 3 mice given 10^7 sensitised cells and 5×10^7 BRBC minus the mean swelling of 3 mice given 10^7 sensitised cells without antigen. The standard error of the difference is represented by the numbers in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.

iv) Antigen-specific nature of the helper cell.

The antigen-specific nature of the helper cell was tested in an adoptive transfer system. This system has been extensively characterised in this laboratory by Dr. Bretscher, and it has consistently been observed that lethally irradiated animals injected with 3 x 10^6 normal spleen cells and 4 x 10^8 xenogeneic red cells do not mount a reliable DTH response to this antigen in 6 days. Mice were given primed helper cells 24 hours before irradiation to see if the presence of specific helper cells in these animals would support the induction of DTH in this limited normal spleen cell transfer. Helper cells were harvested from mice primed sc 6 days previously with either CRBC or BRBC. These were injected at a dose of 10^7 per mouse into several groups of mice as outlined in Table XIX. Twenty-four hours later, the mice were irradiated and injected iv with a limiting dose of normal spleen cells and either BRBC or CRBC as indicated. The response was augmented in an antigen-specific fashion, i.e., the CRBC response was increased over the background level only if CRBC-primed as opposed to BRBC-primed cells were preinjected into the mice. The BRBC response was also increased only in those mice given specifically primed cells. The cells primed with BRBC do not increase the CRBC response at all, and helpers specific for CRBC do not affect the BRBC response. These results are obviously not as clear as one would like to see as a demonstration of *in vivo* help. The DTH response to BRBC is minimal, and the response to CRBC is fairly high in those mice not receiving help. The results are, however, at least suggestive that helper cells for the induction of DTH act in an antigen-specific fashion.

TABLE XIX

An adoptive transfer study indicates that the helper cell for the induction of DTH is antigen-specific.

	normal spleen[1]	helper cells[2]		antigen ($4x10^8$)	swelling[3]		
		BRBC	CRBC		+BRBC	+CRBC	-ag
A)	$3x10^6$	---	---	BRBC	33(4)	N.D.[4]	23 (3)
B)	$3x10^6$	---	---	CRBC	N.D.	67(5)	19 (5)
C)	$3x10^6$	10^7	---	BRBC	62(3)	N.D.	29 (2)
D)	$3x10^6$	---	10^7	CRBC	N.D.	187(6)	61(10)
E)	$3x10^6$	10^7	---	CRBC	25(3)	76(4)	23(10)
F)	$3x10^6$	---	10^7	BRBC	31(6)	21(6)	29 (6)

1) Mice were lethally irradiated with 850 rad. of gamma irradiation, and were injected iv with 3 x 10^6 normal syngeneic spleen cells and 4 x 10^8 RBC as antigen.
2) The helper cells were derived from the spleens of mice primed sc in the footpad 6 days previously with 5 x 10^8 of the appropriate RBC in saline. The helper cells were injected at a dose of 10^7 per recipient mouse 24 hours before irradiation.
3) The swelling activity was measured by harvesting the spleens of the irradiated reconstituted mice after 6 days and injecting 10^7 of these viable cells into the footpad of a naive mouse with 5 x 10^7 BRBC, 5 x 10^7 CRBC or no antigen. Numbers in parentheses represent the standard errors of 3 mice per group.
4) Not done.

v) Summary.

In this section, the addition of primed irradiated helper cells has allowed the induction of DTH reactivity in those cultures containing increased numbers of normal spleen cells and decreased antigen concentrations. More efficient helper cells were obtained from mice primed sc in the footpad with 5 x 10^8 RBC in saline, than from those primed iv with low doses of erythrocyte antigen. FGG-specific helper cells, on the other hand, were more efficiently induced by the iv injection of soluble protein. An adoptive transfer experiment has suggested that the helper T cell acts in an antigen-specific fashion.

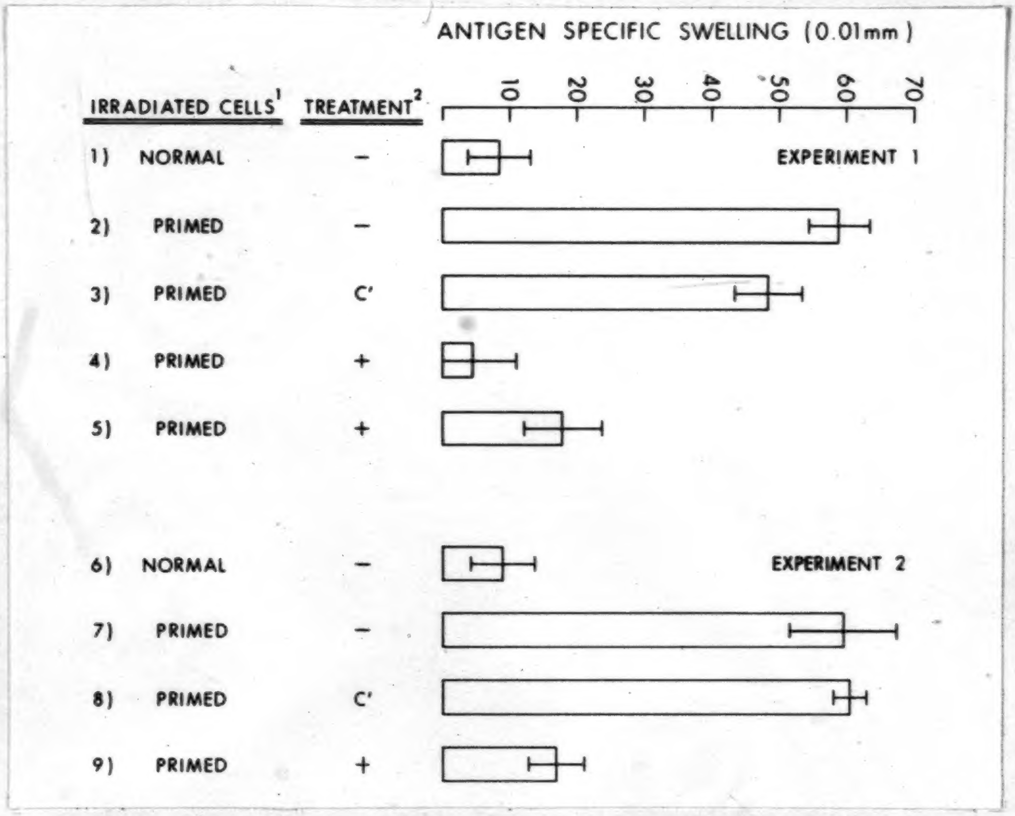
The culture system was now considered reliable for determining the nature of the helper cell and its mechanism of action. Formal experiments were designed to characterise the helper activity and the mechanism of action of this helper population. The experiments in the following section have been designed to provide sufficient cells so that 10 mice per experimental group (5 with, 5 without antigen) can be tested. These formal experiments were repeated a minimum of 3 times each, and equivalent results were obtained. Representative experiments with appropriate control results are presented.

B) Nature of the helper cell for the induction of DTH and its mechanism of action.

1) A Thy-1 bearing cell acts as a helper cell in the induction of DTH.

Spleen cells from mice primed sc 6 days previously with BRBC were used to help induce DTH reactivity in cultures containing 10^7 normal spleen cells and 10^7 BRBC. It can be seen from Figure 2 that DTH is only induced in those cultures which contain primed irradiated spleen cells as opposed to normal irradiated spleen cells (rows 2 vs 1, 7 vs 6). Treatment of the helper population with anti-Thy-1 antiserum and complement before addition to the cultures results in a loss of the induction of DTH (rows 2,4); while treatment of this population with complement alone does not significantly reduce the amount of DTH induced (rows 2,3; and 7,8). Those primed cells that have been treated with anti-Thy-1 antiserum and complement do not produce help when supplemented with normal irradiated spleen filler cells (row 5). This indicates that the anti-Thy-1 antibody treatment is removing a T cell from this primed population which is not present in great numbers in normal spleen, and is consistent with the Thy-1 bearing cell being antigen-specific.

Figure 2



A Thy-1 bearing cell acts as a helper cell in the induction of DTH.

1) Irradiated cells received 1500 rad. of gamma irradiation before being added to the cultures. These came from unprimed mice (normal) or mice primed 6 days previously with 5 x 10^8 BRBC sc in the footpad.

2) The population of helper cells was treated before being added to the cultures with anti-Thy-1.2 antibody and complement (+), (yield approximately 70%), or with complement alone (C'), (yield approximately 83%).

Row 5 is equivalent to row 4 except that normal irradiated spleen filler cells have been added such that 10^7 cells were added per culture.

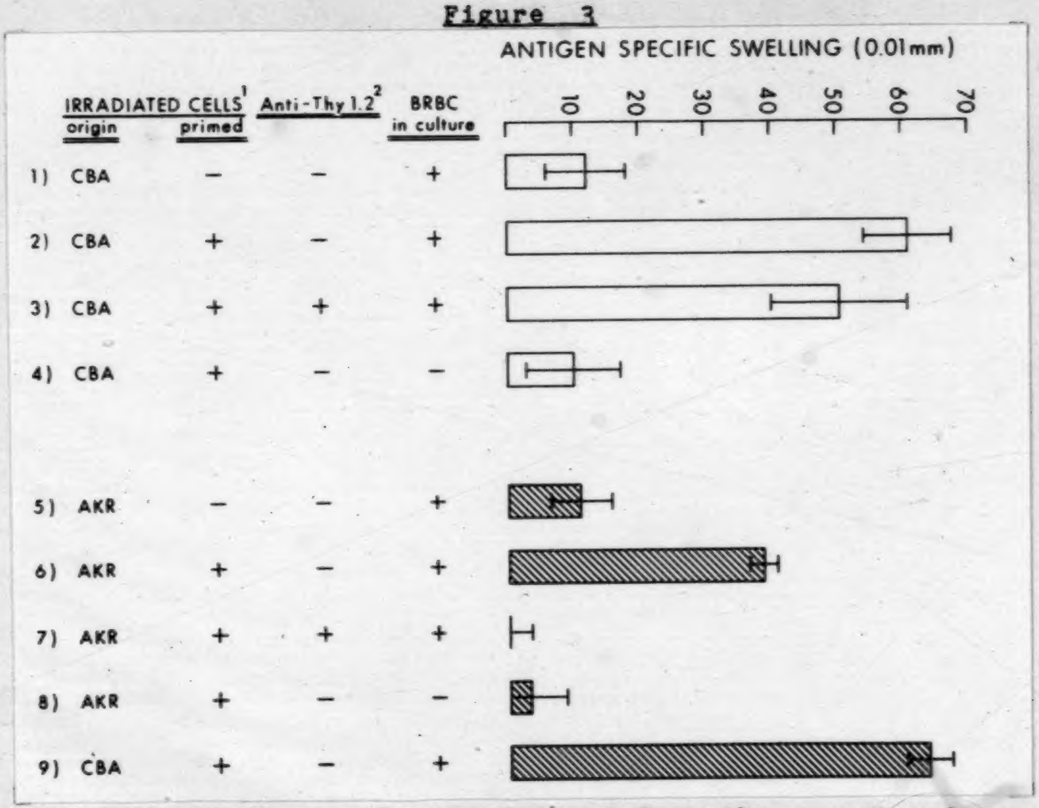
2) The DTH effector cells are derived from the normal spleen cell population.

The possibility existed that the DTH activity observed was actually due to some cells derived from the primed spleen cell population even though this would require them to have survived 6 days in culture after receiving 1500 rad. of irradiation. The Thy-1 cell marker was used to determine which population of cells gave rise to the DTH effector cells. Spleen cells were harvested from two mouse strains which carry the same major histocompatibility antigens, but differ in their Thy-1 alleles, i.e., CBA/CaJ and AKR/J. Mice of one strain were primed, their cells irradiated, and used to cooperate in culture with normal spleen cells of the same or the other mouse strain. At the end of the incubation, the DTH effector cells were treated with antiserum specific for the Thy-1.2, i.e., the CBA/CaJ antigen.

As shown in Figure 3, the antiserum and complement treatment completely eliminated any footpad swelling in those mice given sensitised cells from cultures in which CBA/CaJ normal spleen cells had been incubated with AKR/J irradiated primed spleen cells (rows 6,7), but not in those mice given cells from cultures in which AKR/J normal spleen cells had been incubated with CBA/CaJ irradiated spleen cells (rows 2,3). This shows that the DTH effector cells are derived from the normal spleen cell population, and not from the primed irradiated cells used as a source of helper activity in these experiments.

Control experiments show that allogeneic effects due to minor histocompatibility differences are not responsible for the helper activity of the irradiated population. Thus, when normal cells of one strain are incubated with irradiated normal cells of the other strain in the presence of antigen, no significant antigen-specific swelling is induced (rows 1,5); in contrast, when irradiated primed cells of one strain are incubated with the normal cells of the other strain in the presence of antigen, significant DTH is induced (rows 2,6). Furthermore, the induction of DTH in this non-syngeneic situation still requires the presence of antigen (rows 4,8). Finally, it can be seen from Figure 4 that the DTH induced in cultures containing responder cells of either strain is not significantly different when each is helped by the CBA/CaJ primed irradiated spleen cells (rows 2,9). In conclusion, these experiments demonstrate that the DTH effector cells are derived from the normal spleen cell population.

Figure 3



The DTH effector cells are derived from the normal spleen cell population.

1) Irradiated cells received 1500 rad of gamma irradiation before being added to the cultures. These came from unprimed mice (-) or mice primed 6 days previously with 5 x 10^8 BRBC sc in the footpad (+). These cells were from either CBA/CaJ or AKR/J strains of mice, and were added to cultures containing normal spleen cells from either CBA/CaJ (hatched bars) or AKR/J (open bars).

2) The DTH effector cells generated after 6 days in culture were treated with anti-Thy-1.2 antiserum (specific for the CBA/CaJ antigen) and complement before being injected into the footpads of normal CBA/CaJ mice. This treatment killed 30% of the viable cells after normal spleen cells from AKR/J mice had been cultured with primed irradiated cells from CBA/CaJ mice, and 82.5% after normal spleen cells from CBA/CaJ mice had been cultured with primed irradiated cells from AKR/J mice. These cells were supplemented with normal spleen cells from CBA/CaJ mice such that 10^7 cells were injected per footpad.

3) The helper T cell for the induction of DTH is antigen-specific.

Mice were primed with either BRBC or CRBC and their irradiated spleen cells used as a source of helper cells to induce DTH effector cells from normal spleen cells cultured *in vitro* with either the same or the other red cell antigen. From the results shown in Table XX it can be seen that swelling activity is induced and elicited only when the same red cell antigen is present during the *in vivo* priming, the *in vitro* induction period and elicitation. This demonstrates a lack of cross-reactivity between these two red cell antigens at the level of the helper population and the DTH effector population, and shows that the help is acting in an antigen-specific fashion. Thus the spleen cells from mice primed with BRBC only provide helper activity for the induction of BRBC-specific DTH and only if BRBC are present in the culture system. The mice primed with CRBC only induce CRBC-specific DTH and only if CRBC are present in the culture, and this can only be detected if CRBC are also present in the footpad during elicitation. It can also be seen from Table XX that the presence of BRBC-primed spleen cells and both red cell antigens in culture only results in the induction of BRBC-specific DTH. These results argue against the possibility that a non-antigen-specific long range factor(s), released as a consequence of an interaction between the helper T cell and antigen, allows DTH precursor cells to be induced.

TABLE XX

The helper T cell is antigen-specific

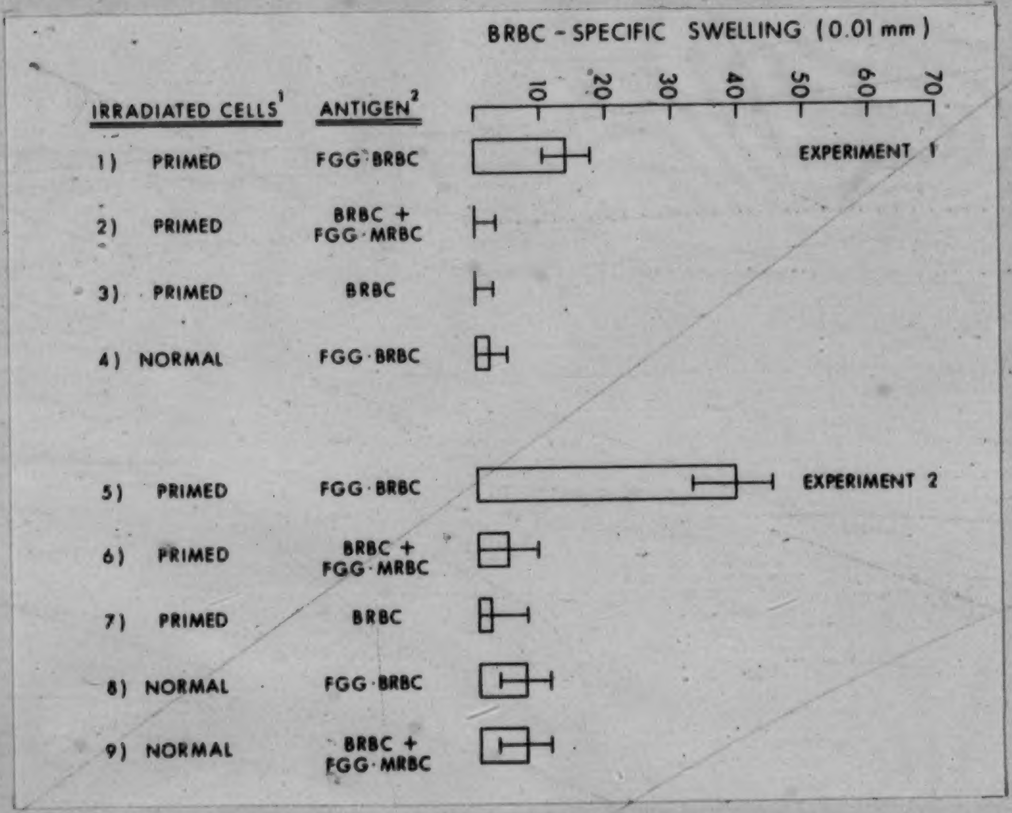
Antigen			Antigen-specific swelling(10^{-2}mm)[4]	
priming[1]	culture[2]	footpad[3]	exp 1.	exp 2.
---	BRBC	BRBC	5 (5)	2 (2)
BRBC	BRBC	BRBC	81 (6)	68 (8)
BRBC	CRBC	BRBC	2 (1)	8 (6)
BRBC	CRBC	CRBC	10 (6)	6 (6)
---	CRBC	CRBC	6 (5)	4 (2)
CRBC	CRBC	CRBC	68 (7)	45 (8)
CRBC	CRBC	BRBC	6 (2)	N.D.[5]
CRBC	BRBC	CRBC	-9 (7)	10 (3)
CRBC	BRBC	BRBC	0 (1)	11 (3)
BRBC	BRBC+CRBC	BRBC	72 (7)	N.D.
BRBC	BRBC+CRBC	CRBC	13 (7)	N.D.

1) Animals were primed sc in the footpad with 5 x 10^8 of the appropriate RBC 6 days before their spleens were removed and used as a source of helper cells.
2) Cultures contained 10^7 normal spleen cells and 10^7 of the designated RBC as antigen.
3) 10^7 viable harvested cells were injected per footpad with 5 x 10^7 of the designated RBC as antigen.
4) Antigen-specific swelling is calculated as the mean swelling (in 10^{-2}mm. units) of 5 mice given 10^7 sensitised cells and 5 x 10^7 RBC minus the mean swelling of 5 mice given 10^7 sensitised cells without antigen. The standard error of the difference is represented by the numbers in parentheses, and is calculated as the square root of the sum of the squares of the standard errors with and without antigen.
5) N.D. = not done in this particular experiment.

4) The helper T cell for the induction of DTH acts via linked recognition.

If helper T cells act via linked recognition, helper cells specific for one antigen would allow the induction of DTH specific for the other antigen only when the conjugate was present in the cultures. FGG-specific helper cells were obtained from the spleens of mice injected with 0.5 mg. of FGG iv 2 or 4 days previously. These cells were irradiated and tested to see if they would cooperate with normal spleen cells to induce BRBC-specific DTH in cultures containing either the conjugate FGGBRBC or BRBC plus the conjugate FGGMRBC. The results shown in Figure 4 demonstrate that BRBC-specific DTH is induced only when FGGBRBC is provided as antigen in cultures containing FGG-specific helper cells (rows 5,1). Although the helper cells obtained after 2 days of in vivo priming with FGG allow the induction of a small antigen-specific swelling (significantly different from the control groups, i.e., rows 1 vs 2,3,4, $P<0.005$ by Student's t test), the cells obtained after 4 days of priming provide sufficient help to induce a good DTH response in those cultures which contain the FGGBRBC conjugate as antigen. BRBC-specific DTH is not induced when both BRBC and FGG (linked to MRBC) are present in the cultures, i.e., physical linkage between the antigen against which the helper T cells are raised and the antigen inducing the DTH precursor cell is required (rows 5 vs 6, 1 vs 2). As can be seen from rows 5 versus 8, the conjugate requires the presence of FGG-primed cells in order to induce DTH to BRBC.

Figure 4



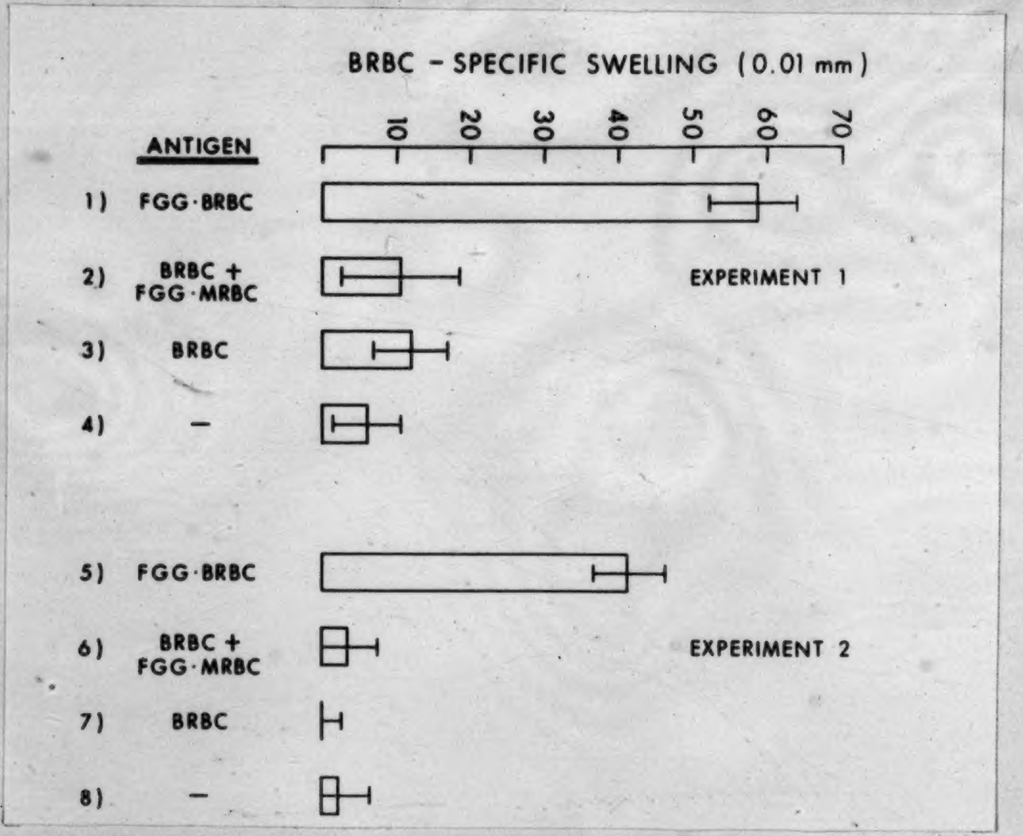
Irradiated helper T cells for the induction of DTH act via linked recognition.

1) Irradiated cells received 1500 rad. of gamma irradiation before being added to the cultures. These came from unprimed mice or mice primed iv 2 (Experiment 1) or 4 (Experiment 2) days previously with 0.5mg. of soluble FGG in saline.

2) The conjugated antigens were prepared by incubating specific fowl-anti-RBC antibody at a 2-fold lower than hemagglutinating concentration for 30-60 minutes at 37°C. BRBC are present in all cultures, either linked to FGG, or not, and the DTH is measured against BRBC.

In these experiments the mice are primed with one antigen and the DTH is induced against another non-cross-reacting antigen and it was therefore not necessary to irradiate the helper population as the FGG-primed cells did not give rise to BRBC-specific DTH when incubated with BRBC. The FGG-primed cell population could be used as the source of BRBC-specific DTH precursor cells. When these cells are incubated at 10^7 cells per well with 10^7 RBC, reliable BRBC-specific DTH was induced only in those cultures which contained the FGGBRBC conjugate as antigen (Figure 5). The unirradiated cells from mice primed 2 days previously with FGG are seen to provide very effective help for the induction of BRBC-specific DTH, even though they did not provide very efficient help when irradiated. The unirradiated cells from the mice primed 4 days previously with FGG induce BRBC-specific DTH in cultures not significantly different from that induced in normal spleen cells that are complemented with irradiated FGG-specific helpers. These observations show that the FGG-specific help obtained after 2 days of *in vivo* priming is relatively sensitive to irradiation compared to the help obtained after 4 days of priming, and is expected if the 2 day, as opposed to the 4 day, primed cells have to divide or further differentiate to provide adequate helper function.

Figure 5



FGG-specific helper cells for the induction of BRBC-specific DTH act via linked recognition.

These FGG-specific cells are the same cells used in the experiments reported in Figure 4, except that these are now acting as a source of BRBC-specific precursor cells, as opposed to helping induce a population of normal spleen cells. The FGG-specific cells were not irradiated in these experiments. The FGG-specific helper cells are from mice primed iv 2 (Experiment 1) or 4 (Experiment 2) days previously with 0.5mg. of soluble FGG in saline.

5) Summary

The results in this section have shown that the limiting system developed can be helped to induce a DTH response by antigen-specific, Thy-1 bearing, radioresistant, helper cells derived from a mouse primed to mount a DTH response. The antigen-specific swelling observed is not due to the helper population, but to the induction of cells present in the normal spleen population. The mechanism of action of these helper cells is the linked recognition of the antigenic determinants recognised by the primed irradiated helper cell and the DTH precursor cell. There is no non-specific component of this response noted at any stage - the correct (identical or linked) antigen is necessary for priming, induction and elicitation.

DISCUSSION

The purpose of this study was to determine the requirements for the induction of DTH *in vitro* and to study the mechanism by which cellular cooperation functioned in this induction. Numerous experimental systems were tested to find conditions which would result in DTH induction *in vitro* and the most critical factors were shown to be the density of normal spleen cells and the concentration of antigen present. In fact, moving from one culture system to another, one could predict the optimum conditions for the induction of DTH by relating the surface area of the new culture system to the old, and ensuring that the new cultures would contain the same number of responding cells and antigen per unit surface area. The practical differences between systems were survival rates of the cells, and ease of handling. The assay system employed required so many viable cells at the end of a 6 day culture period that survival rates really governed which experimental system would be used for the bulk of these studies. Thymocytes can be induced to DTH reactivity, but do not survive well in culture and for this reason were not practical as a source of responder cells. Spleen cells do survive well and these were used throughout nearly all of the experiments reported here, using Costar culture dishes because these were the easiest to handle in large quantities.

Observations from the study of varying responder cell density and antigen concentrations in culture (Figure 1) suggest that 10^7 normal spleen cells contain sufficient antigen-specific precursor cells to generate measurable DTH reactivity since these respond to high antigen concentrations (10^9 RBC/culture), but that these are not induced when cultured with lower antigen concentrations (10^7 RBC/culture). Those cultures that contain just one-half the number of normal spleen cells that give a response with the high antigen concentration are not induced. Each of these culture conditions that do not, on their own, allow the induction of antigen-specific DTH can be helped by the addition of antigen-specific, primed, Thy-1-bearing cells whose activity is radioresistant. These helper cells are best detected in mice primed in a manner that induces DTH to the relevant antigen in the whole animal. It was not possible to consistently demonstrate efficient help for a DTH response with T cells derived from a mouse mounting a humoral response. This may be because the two helper cells are different or because another regulatory cell is present under conditions that lead to a humoral response which inhibits the induction of DTH. It appears that the question of whether the same T cell can help in the induction of all classes of immune response can most clearly be resolved by studies with cloned, stable helper T cell lines, or biochemically purified, antigen-specific helper factors, as opposed to populations of primed spleen cells.

The studies described here have clearly shown that the antigen-specific swelling observed is induced in the normal spleen cell population. The irradiated helper cells from antigen-primed mice are necessary for the induction, but not for the elicitation of the response. It has also been shown that the helper cell acts in an antigen-specific fashion. Those cultures containing both BRBC and CRBC with helper T cells from BRBC-primed mice do not induce any DTH specific for CRBC (Table XX). This observation shows that the helper T cells do not act *in vitro* by releasing a non-specific, long range factor, but does not bear on whether the helper T cell receptors are specific for the antigen or for idiotypes on BRBC-specific DTH precursor cells. The fact that DTH reactivity against a second antigen (BRBC) can be induced, but only if this second antigen is physically linked to the antigen with which the helper T cells were primed (FGG), argues against the helper T cells examined here having anti-idiotype specificity. These experiments with separate or conjugated antigens again emphasise that the helper T cells act only over a very short range since cultures containing the same two antigens induce DTH reactivity against BRBC only when the FGG is physically linked to the BRBC. It is not sufficient for the induction of BRBC-specific DTH to have BRBC present in the cultures, FGG linked to MRBC, and FGG-primed helper cells.

These studies employing FGG-primed helper cells show that triple complexes of antigen, antigen-specific helper T cell (or helper T cell factor) and the antigen-specific precursor cell must be formed to induce DTH reactivity. This would be consistent with the requirement being for two signals (one from antigen, and one from antigen-specific help) for the induction of DTH precursor cells. This requirement provides an explanation for the observations described in Figure 1 on the effect of varying responder cell density and antigen concentration on the induction of DTH. As the density of normal spleen cells is increased, the density of BRBC-specific immunocompetent cells increases and effective cooperation will occur if sufficient antigen is provided to ensure that two antigen-specific cells, in close proximity to one another, can form triple complexes. Thus a higher density of spleen cells can support the induction of DTH in the presence of a lower concentration of antigen than can a lower density. This semi-quantitative interpretation is supported by the observation that a low density of cells (10^7) cultured in the presence of a moderate concentration of antigen (10^7 RBC) will only support the induction of a strong DTH response if supplemented with antigen-specific helper T cells. Similarly, an even lower density of spleen cells (5×10^6) cultured in the presence of a high concentration of antigen (10^9 RBC) will only support the induction of a strong DTH response if supplemented with antigen-specific helper T cells. It has also been shown, both during the course of this study and elsewhere (Bretscher, 1979), that the lower density of cells (10^7) can result in strong DTH reactivity after incubation with 10^7 erythrocytes if the responder cells come from an antigen-primed animal. One interpretation would be that this activity is induced because the specific antigen priming increases the frequency of antigen-reactive cells. These findings, together with those that show helper T cells act by linked recognition, provide a reasonable quantitative basis for understanding how the spleen cell densities and the antigen concentrations are interdependent in the way they affect the induction of DTH.

A few studies have been reported here concerning the induction of humoral immunity in culture, and the trend seen is that higher densities of normal spleen cells tend to induce more humoral immunity and less DTH reactivity (for a given antigen concentration) than do lower densities. Extending the ideas developed above, this shift in immune reactivity could be due to increased numbers of cooperative events in the inductive phase. This would be consistent with observations by other workers. The *in vitro* culture system employing a single spleen cell density and varying antigen concentrations described by Ramshaw and Eidinger (1979), induces only DTH at a low antigen (HRBC) concentration, only PFC at a higher antigen concentration, and only DTH again at the highest antigen concentrations tested. The fact that DTH is induced again at very high antigen concentrations can be interpreted as a requirement for two antigen-specific cells to be recognising the same physical particle or physically linked determinants, in order to induce immune reactivity. At very high antigen concentrations, the antigen-specific cells would be saturated with antigen such that fewer pairs would recognise the same antigenic particle. Thus, fewer cooperative events will be initiated at extremely high ratios of antigen to normal spleen cells. Other work includes an in vitro culture system employing a single antigen concentration (BRBC or CRBC) and varying the normal spleen cell densities (Bretscher, manuscript in preparation). Again, the immune response induced shifts from none, to only DTH, to only PFC as the density increases. The same pattern of response occurs in the adoptive transfer system (Bretscher, manuscript submitted), i.e., as the amount of normal spleen transferred increases and the antigen (BRBC or CRBC) dose remains the same, the response shifts from none induced to only DTH to only PFC.

The basic pattern is just that observed by Pearson and Raffel (1971) when they emphasised that the same antigenic determinants could induce DTH or antibody responses in vivo under appropriate conditions. They suggested that the conditions which favour the induction of DTH reactivity are those in which the antigen is "not very foreign" either because of its small size or its similarity to self. This could be because of the decreased numbers of antigen-specific cells for such antigens which would result in decreased numbers of cooperative events for the induction of immune reactivity against these antigens. The same argument could also apply to the preferential induction of cell-mediated immunity following immunisation with low amounts of an antigen which contains more foreign sites.

Experiments in which induction of several classes of immune response against the same antigen occurs by merely adjusting the quantities of responder cells and/or antigen, allow one to examine the cellular basis of immunoregulation. Depletion of particular cell types or enrichment of others in the experimental protocol can determine the cells responsible for induction and for the class of response induced, and even those responsible for switching the class of response from one type to another. Cells responsible for "class switching" or, more precisely, for inhibiting the induction of the expected class of response following a particular immunisation protocol have been characterised in some *in vivo* experiments using HRBC as antigen (Ramshaw, Bretscher and Parish, 1976,1977). Spleen cells from mice mounting a humoral response, transferred to a syngeneic mouse subsequently immunised to induce a DTH response, will inhibit the induction of DTH to that antigen and determinants physically linked to that antigen (Ramshaw, Bretscher and Parish, 1976). Similarly, spleen cells from mice mounting a DTH response, transferred to syngeneic animals subsequently immunised to develop a humoral response, will inhibit the induction of a humoral response to that antigen (Ramshaw, Bretscher and Parish, 1977). These inhibitory cells were characterised as T cells and carried specific surface antigens that allowed them to be distinguished from one another, i.e. they were class-specific inhibitory T cells (Ramshaw, McKenzie, Bretscher and Parish, 1977).

Antigen-specific cells that inhibit the induction of one class of immunity tend to be found during a strong response of another class against the same antigen. Transfer of nylon-wool purified (T) cells from the spleens of mice mounting a humoral response against BRBC or CRBC can switch the induction of an expected DTH response to a humoral one. These cells are transferred to mice 24 hours before irradiation. A normal spleen cell and antigen inoculum which usually results in the induction of DTH is then transferred to these animals and results in the induction of a humoral response instead, in an antigen-specific fashion (Bretscher, manuscript submitted). The same is true for the transfer of such T cells to cultures containing a normal spleen cell density that normally results in the induction of DTH (Bretscher, manuscript in preparation). Thus, if nylon-wool purified T cells from a mouse mounting a humoral response to CRBC are added to cultures that would normally result in a DTH response to CRBC, a PFC response is induced and no DTH against CRBC. These cells do not affect cultures that would normally result in a DTH response to BRBC. An analogous antigen-specific effect is seen for purified irradiated T cells from a mouse mounting a humoral response to BRBC. Furthermore, the effect can be titrated with the number of irradiated primed T cells added to the cultures and, as more T cells are added, the DTH response decreases and the PFC response increases.

It is not yet known whether class-specific, inhibitory, antigen-specific T cells are generated in normal spleen cell cultures concomitantly with a response of another class, but this could be tested. T cells which inhibit the induction of a humoral response in an antigen-specific fashion, have been demonstrated concomitantly with DTH effector cells in spleen cell cultures from mice primed iv 3 days previously with a low dose (10^5) of HRBC (Ramshaw, Woodsworth, Wright and McKenzie, 1980). It is also not yet known whether such "suppressor cells" could inhibit an ongoing response to a particular antigen, and this should be examined. If it is possible to generate cells in culture which would specifically inhibit a particular class of immune response in an antigen-specific fashion, and if one could separate these from the other antigen-specific cells in culture, one could apply this to experimental models of autoimmunity, e.g., experimental allergic encephalomyelitis (EAE). This autoimmune disease, often regarded as an experimental model for multiple sclerosis, is thought to be due to DTH reactivity against a protein found in the myelin sheath surrounding tissue of the central nervous system (Arnon, 1981). If one could induce potent DTH reactivity to this antigen *in vitro*, one might induce disease by injecting these cultured cells into animals. This would establish whether or not the disease symptoms are genuinely due to DTH reactivity against this particular protein. By varying culture conditions, and inducing humoral immunity against the same antigen, one might produce antigen-specific T cells which could inhibit this DTH reactivity, and determine whether this would then eliminate disease symptoms. It would be extremely useful in many instances to be able to specifically inhibit certain immune reactivities, especially in patients suffering from autoimmune disorders or in transplant recipients; or to be able to switch the class of immune response induced against a particular antigen from a detrimental one (e.g. IgE against ragweed pollen) to a relatively harmless one (e.g. IgG). Any knowledge that can be gained by using experimental models to understand immune regulation should be useful in the eventual treatment of patients with these disorders.

The particular system developed during the work described in this thesis could be used to study other aspects of immune regulation. A help-limiting system for the induction of DTH could be used to determine whether the same helper cell or helper factor that induces humoral immunity can help to induce DTH reactivity. Biochemically purified helper factor (from syngeneic animals) could be titrated to see if it might also induce DTH reactivity in this help-limiting system. This would be one way to determine whether help is class-specific itself or whether the quantity of antigen-specific help (or helper factor) available determines the class of immune response induced. A very limited attempt to induce DTH in this system using a BRBC-specific helper factor for humoral responses purified by Dr. C. Shiozawa of this department did not result in the induction of DTH, but this should be tested thoroughly with an extensive dose response titration and complete kinetic studies.

Other aspects of the two-signal model for the induction of T cells could also be tested in this system. DTH can be induced in vitro, and the induction of DTH requires antigen-specific help. This means that help must also be generated in vitro, and should develop before the DTH activity appears. In fact, when cultures that were set up under conditions that would induce DTH reactivity in 6 days, were harvested and irradiated after 4 days, they provided very efficient help for the induction of DTH in subsequent help-limiting cultures. Unfortunately, this help was not tested for antigen-specificity in this particular instance. However, the antigen-specific nature of this help could be tested, and, assuming it is antigen-specific, the conditions for the generation of this help could be studied. To test whether the induction of help in the first cultures requires cellular collaboration (2 signals), one could make the first cultures limiting for the induction of help by dropping the normal spleen cell dose until no help is induced. Similar conditions could then be used to determine whether the addition of specifically primed, irradiated helper cells as opposed to irradiated cells primed to a different antigen, would support the induction of antigen-specific help in these first cultures. A few preliminary attempts to test this induction of help indicate that normal spleen cells originally cultured with BRBC-primed irradiated spleen cells and BRBC only help induce a BRBC-specific DTH response in subsequent cultures and, similarly, CRBC-specific helper cells can also be induced.

Helper T cells can be induced in vivo since primed, as opposed to normal, spleen cells can provide specific helper function. It seems likely that helper T cells for the induction of DTH are also generated under those conditions of in vitro culture that lead to the production of DTH reactivity. These considerations stress that the helper T cells characterised here may act indirectly in aiding the production of DTH precursor cells by, for example, aiding the induction of more helper T cells. These observations do not bear on whether one or more sequential cellular interactions occur in these cultures; however, if several occur, they all must act by the linked recognition of antigenic determinants as one interaction, mediated by a long range non-specific factor, would obviate the need for the linked recognition observed in these studies.

The theory concerning the 2-signal model of induction and its explanation for self-tolerance (Bretscher and Cohn, 1970) also predicts that immunocompetent precursor cells incubated under very dilute culture conditions might very well result in antigen-specific tolerance, if this occurs by one signal alone. Conditions would have to be such that the cultured cells would be unlikely to be able to collaborate efficiently, but would be in the presence of sufficient antigen that all antigen-specific precursor cells would be in contact with the antigen. This could be tested by harvesting sparse cultures exposed to antigen, adding antigen-specific primed, irradiated helper cells, and testing for the ability of these cells to generate DTH effector cells. Controls must include cells preincubated with other antigens for specificity, and higher density cultures preincubated with the same antigen to ensure the tolerance (if it occurs) is consistent with its being due to lack of cooperative events in the first cultures.

The system developed in this thesis also provides a means by which the H-2 restriction of T cells, other than cytotoxic T cells or those involved in the thymidine incorporation assay, can be studied *in vitro*. One might test the restriction of T-T cooperation in primed or normal cells for comparison to T-B, T-macrophage, or B-macrophage restriction.

In conclusion, I would like to say that the system developed in this thesis may be used to answer some important current questions in the field of immunoregulation, especially whether the 2-signal model for induction applies to immunocompetent precursor T helper cells for the induction of DTH, and whether the help required for induction is the same for all classes of immunity. Clearly, the more one understands about immune regulation, the better position one is in to modify the response at will, helping to induce appropriate responses when these are lacking or eliminating harmful or inappropriate immune responses. The work described here on the requirements for induction of a DTH response, together with that done by Dr. Bretscher on the switching of the class of response induced *in vitro* from DTH to humoral, suggest that the number of cooperative events occurring in a culture, is the single most important factor in determining whether any, and which class, of immune response will be induced. Together, these systems provide a powerful means of studying the immunoregulation of the classes of DTH, IgM and IgG.

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